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**TOPICAL NEGATIVE PRESSURE THERAPY IN WOUND HEALING:
A RESEARCH TOOL TO STUDY NEUTROPHIL-MEDIATED
WOUND PATHOPHYSIOLOGY IN ACUTE DERMAL WOUNDS**

Titus Sam Turner Adams

A dissertation submitted to the University of Bristol in accordance with the requirements of the degree of Doctor of Medicine in the Faculty of Medicine.

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ABSTRACT

Topical Negative Pressure therapy is in widespread use in the management of acute and chronic cutaneous wounds. The mechanisms of action are not fully understood, but are likely to be multifactorial. Experience of this therapy is based on a number of clinical series, case reports and some animal studies. There is a lack of direct evidence to determine its mechanisms of action and to support its clinical efficacy in human wound healing. This problem stems partly from difficulties in applying selective negative pressure to open exudative wounds. A new approach was required in applying this technology to wound surfaces in consenting patients.

This thesis describes the design and validation of 'standard' and 'irrigation' devices that were used to apply Topical Negative Pressure to one part of a wound thus allowing intra-patient control. As a novel research tool, the irrigation device provided an opportunity to collect wound fluid from the surface of the wound for biochemical analysis. Paired wound biopsies of Topical Negative Pressure treated and control wounds were obtained.

This thesis has demonstrated that Topical Negative Pressure (with intermittent suction) modulated the acute donor site wound and partial thickness burn wound during the first 48 hours of injury, by altering the distribution of inflammatory neutrophils in the dermis.

Using a cycle of periods of suction-on and suction-off, Topical Negative Pressure increased the removal of Neutrophil Elastase from the wound during suction, in addition to its endogenous plasma-derived inhibitor, α_1 -Protease Inhibitor.

Using this new device on human wounds has provided a means of understanding mechanisms in Topical Negative Pressure therapy, and has demonstrated its use as a research tool in the collection and analysis of wound fluid.

This thesis is dedicated to my wife, Arabella

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AUTHOR'S DECLARATION

I declare that the work in this dissertation was carried out in accordance with the regulations of the University of Bristol. The work is original except where indicated by special reference in the text and no part of the dissertation has been submitted for any other degree.

Any views expressed in the dissertation are those of the author and in no way represent those of the University of Bristol.

The dissertation has not been presented to any other University for examination either in the United Kingdom or overseas.

SIGNED:

A handwritten signature in black ink, consisting of a stylized 'M' followed by a horizontal line that curves upwards at the end.

DATE: 26. 11. 05

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LIST OF ABBREVIATIONS

7-AMC	7-amino-4-methylcoumarin
α_1 -PI	α_1 -protease inhibitor
ARDS	Acute Respiratory Distress Syndrome
AP	Alkaline Phosphatase
AWF	Acute wound fluid
CAMs	Cellular adhesion molecules
CG	Cathepsin G
CWF	Chronic wound fluid
CyA	Cyclosporin A
ddH ₂ O	Double distilled water
DAB	3,3 Diaminobenzidine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DPX	Dibutyl Phthalate and Xylene
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
ESI	Elastase specific inhibitor
FDP	Fibronectin degradation products
fMLP	formyl-methionyl-leucyl-phenylalanine
H&E	Haematoxylin and Eosin
HUVEC	Human Umbilical Vein Endothelial Cells
IL	Interleukin
LDPI	Laser Doppler Perfusion Imaging
LTB ₄	Leukotriene B ₄
MMP(s)	Metalloproteinase(s)
MoAbs	Monoclonal antibodies
MOF	Multiple Organ Failure
MT	Masson's Trichrome
NE	Neutrophil Elastase
PA	Plasminogen Activator
PAF	Platelet Activating Factor
PAS	Periodic-Acid Schiff
PBS	Phosphate Buffered Saline

PMA	Phorbol Myristate Acetate
PMSF	Phenylmethanesulfonylfluoride
PUE	Polyurethane Ester
PVA	Polyvinyl Alcohol
ROS	Reactive Oxygen Species
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
SERPIN	Serine Protease Inhibitor
SKALP	Skin-derived antileucoprotease
SLPI	Secretory leucoprotease inhibitor
TBSA	Total body surface area
TIMP(s)	Tissue Inhibitor of Metalloproteinase(s)
TNP	Topical Negative Pressure
V.A.C.™	Vacuum Assisted Closure

1 LITERATURE REVIEW

1.1 Topical Negative Pressure Therapy

1.1.1 Definition and History of Topical Negative Pressure

Topical Negative Pressure has been defined as the application of subatmospheric pressure to an open-cell screen (foam, felt, surgical towel or gauze) over a wound bed or skin graft, which is covered by an overlying adhesive polymer drape that creates and maintains a hermetic seal (Greer, 2000). Controlled subatmospheric pressure is applied to this screen using a tube connected to an external suction source. A distinction must be made between the use of topically applied pressure dressings described above, and a closed wound suction system that is commonly employed to coapt tissue surfaces and drain surgical wounds of blood or tissue fluid (Fox and Golden, 1976).

The concept of applying suction to a surface wound is not new. Greer (2000) has reviewed the history of Topical Negative Pressure dating back to Junod's application of suction glass bells in 1841, to create what was described as *therapeutic hyperaemia* (Greer, 2000). In this paper, Greer also cited the use of subatmospheric pressure therapy by a number of international authors who have published work dating back from 1966 (in the Russian literature) to the present, employing commercially available systems. Despite having the Russian papers translated for him, Greer did not provide descriptions of how Russian 'vacuum therapy' had been employed on wounds, the nature of the dressings used, or what results were obtained.

Today, there still remains some confusion within the literature about the use of this form of therapy, its description and application. There are three reasons for this confusion: (1) there are a number of published articles in the world literature in many different languages that cite the use of 'vacuum therapy' in the treatment of wounds. It is difficult to gain access to translated manuscripts; (2) many terms have been introduced to describe this form of 'vacuum therapy'; (3) there are

many different ways of applying 'vacuum therapy' to wounds using a number of different materials which confounds this problem.

1.1.1.1 Terminology

In addition to the commonly used term *Topical Negative Pressure* (Cooper et al., 2000; Deva et al., 2000; Evans and Land, 2001; Shaer, 2001), other generic synonyms include *subatmospheric pressure dressing* (Greer, 2000), *negative pressure therapy* (Banwell et al., 1998), *negative pressure dressing* (Blackburn et al., 1998; Masters, 1998), *vacuum sealing technique* (Fleischmann et al., 1995; Müllner et al., 1997), and *vacuum-assisted closure* (Argenta and Morykwas, 1997a; De Lange et al., 2000). For the purposes of clarity in this thesis, the term Topical Negative Pressure (TNP) will be used to describe all the generic forms of this therapy.

1.1.1.2 Materials and methods used in TNP therapy

In 1986, Shein et al. described the use of a 'sandwich technique' in the management of the open abdomen, in which suction tubes are placed between an inner Marlex® mesh (adjacent to the viscera) and an outer Op-site® dressing, in the open management of major intra-abdominal sepsis (Schein et al., 1986). They illustrated its use in three case studies and mentioned the prevention of bowel evisceration, the reduction of insensible fluid losses, the accuracy of fluid replacement, and the ease of nursing care, as advantages over traditional methods of open wound management.

Smith et al. (Smith et al., 1997) have described their four-year experience (from 1992 to 1996) of a vacuum pack technique of temporary abdominal closure, first described by Brock (Brock et al., 1995). With suction pressures between 100 to 150mmHg, a surgical towel was placed around suction tubes sandwiched between an inner plastic drape and an outer adhesive dressing in 171 general surgical and trauma cases ranging in age from 3 months to 88 years.

In seven case reports, Nakayama (Nakayama et al., 1990) described a technique of applying suction to an adhesive drape to skin grafts of the body, including the hands (Nakayama and Soeda, 1991). These case reports were descriptive

accounts only, and did not include evidence for improved graft take versus any control groups.

In 1993, Fleischmann et al. were the first to publish (in German) the use of a vacuum sealing technique, using foam as an interface (Fleischmann et al., 1993). Their new TNP system involved the use of layers of sterile open-porous polyvinylalcohol (PVA) foam surrounding two redon-drains (16Ch) along the long axis of the foam. The foam was cut to size, and the tubing drawn through the skin. The entire area was covered with a semi-permeable polyurethane drape connected to vacuum bottles. They described its use on 15 patients with open fractures in 1992, as an adjunctive 'sealing' procedure prior to definitive closure by a number of operative techniques. This work, in conjunction with a further 166 cases of acute soft tissue injuries, and acute and chronic wound infections from 1992 to 1993 were later published in English in the European Journal of Orthopaedic Surgery & Traumatology (Fleischmann et al., 1995). They claimed that this technique had been in use in their surgical practice since 1987. With pressures maintained up to a constant suction of 80Kpa (600mmHg), they detailed the types of wounds used with this technique, documented the average time of vacuum sealing, the number of dressing changes used and the subsequent surgical outcomes required to definitive closure. Their work was presented in Germany, at the European Bone and Joint Infection Society Meeting in October 1993.

Meanwhile, Louis Argenta and Michael Morykwas were working on their version of vacuum therapy using a similar open-cell foam dressing since 1989 (unpublished data, Kinetic Concepts, Inc.). The design of their TNP system was slightly different. The foam was made of polyurethane ester (PUE) with greater pore sizes (400µm to 600µm) to maximise tissue growth. This also allowed for reduced subatmospheric pressures of 125mmHg to achieve a uniform collapse of the foam. These authors presented their methodology and basic animal studies using the technique as early as April 1993 in New Orleans, USA and the outcomes of their clinical series at the European Tissue Repair Society meeting in Amsterdam, in August 1993. Argenta and Morykwas filed the technique of 'Wound treatment employing reduced pressure' for patency in March 1993 (Argenta and Morykwas, 1997b). The Food and Drug Administration granted approval for its use in the

treatment of wounds in 1995. The technology has been licensed to and commercially available from Kinetic Concepts, Inc (KCI), and the system trademarked as 'Vacuum-assisted Closure' (V.A.C.™) (Kinetic Concepts Inc., 1996). V.A.C. dressings include sterile PUE foam, an evacuation tube and a transparent adhesive drape. The foam can be trimmed to the necessary size and shape to conform to each individual wound. The evacuation tubes are placed within the foam, and the foam dressing covered with the adhesive drape, extending beyond the wound margins to adhere to dry, normal skin to create an airtight seal. The tubing is attached to a separate V.A.C. canister that is housed within a V.A.C. pump. The pump can be preset to regulate subatmospheric pressures between 25 – 200mmHg, and a sensor detects leaks and provides a warning sound to the operator (Kinetic Concepts Inc., 2000).

From the work by Argenta and Morykwas (1997), the use of suction dressings and a foam interface coupled to a regulated subatmospheric pressure (V.A.C.™) system has been widely accepted and commonly quoted in work related to this method of wound management (Argenta and Morykwas, 1997a). To avoid confusion in this thesis, hereinafter, the term TNP refers *directly to* sealed foam dressings used in conjunction with subatmospheric pressure.

1.1.2 TNP therapy in the Clinical Setting

1.1.2.1 Acute wounds

Fleischmann et al. (1995) mentioned the use of their TNP system as a 'sealing procedure' in a series of patients with 63 acute soft tissue damaged wounds and 47 acutely infected wounds. They described the application of PVA foam (with negative pressures of up to 600mmHg) and length of time of TNP therapy (14.7 and 12.4 days respectively) before further management by delayed primary closure, skin grafting, flap transfer or 'open' treatment. No comment was made about the indications or timing of wound closure when compared to other (chronic) wounds. They observed rapid formation of granulation tissue following TNP in

these acute wounds, but did not document or measure this finding (Fleischmann et al., 1995).

Argenta et al. (1997) published a descriptive series of 300 patients, in whom 88 underwent TNP therapy for the treatment of a variety of acute traumatic and acutely infected wounds. 125mmHg was applied to the foam continuously for 48 hours and then intermittently at 5-minutes-on/2-minutes-off intervals. Rapid rates of granulation tissue formation were observed in acute wounds when compared to non-controlled chronic wounds (Argenta and Morykwas, 1997a). Similar to Fleischmann's report (Fleischmann et al., 1995), they observed that the formation of granulation tissue was most pronounced in acute wounds, and that rate of healing (measured by time to complete healing or the reduction in wound volume) occurred more quickly in this group than the chronic wound groups. No specific details were given in this paper to support these findings. No measurements of granulation tissue formation were made. The 'majority' of the patients either healed completely or until an adequate bed of healthy granulation tissue had developed, and of these, their definitive treatments included grafting, flap or direct closure procedures.

Müllner et al. (1997) summarised the treatment of 12 patients with acute soft tissue traumatic defects. TNP dressings were applied and continuous (undisclosed) subatmospheric pressures maintained with the use of vacuum bottles. Ten out of the 12 wounds were clinically infected. Three patient wounds granulated without secondary closure and nine required skin grafting once sufficient granulation tissue was achieved. The mean healing times were 16 days. The authors suggested that the TNP dressings maintained a clean wound bed and facilitated the earlier application of skin grafts in these wounds, although no objective evidence was presented to substantiate these claims (Müllner et al., 1997).

In these three prospective series, there was no attempt to randomise any of the acute wounds for TNP therapy with matched control groups. The investigators did not specify any reasons for not introducing controls in their studies.

Genecov et al. (1998) applied TNP to 15 human acute donor site wounds to study the rate of reepithelization. Grafts were harvested using a dermatome from both thighs in all but 2 patients, in whom the donor sites were located on the same thigh

with an area of intact skin between the sites. Following grafting, the donor sites were covered with adrenaline-soaked pads, punch biopsies obtained, and one thigh donor site covered with Opsite, and the other with TNP dressings (or half the sites for 2 patients with unilateral donor site wounds). 125mmHg was applied via the V.A.C. pump continuously for up to 7 days. Further biopsies were taken at 4 and 7 days. Biopsies were fixed, sectioned and H&E stained, and a blinded evaluation performed by a dermatopathologist. The degree of reepithelization was determined on a scale of 0 (no evidence of reepithelization) to 4 (complete, mature epithelium). Of the 10 patients who completed the study, seven were noted to have reepithelized faster with the TNP dressings than the Opsite-treated controls by day 7. Two patients showed no difference and one patient showed more reepithelization in the Opsite-treated site. The authors did not provide any information on the histological quantification used in their study in order to categorise them into one of the five groups mentioned. Despite this, this study is the only published human study using TNP dressings against an intra-patient control group (Genecov et al., 1998).

TNP has been used in the effective treatment of acute degloving injuries of the extremities and published as case reports (DeFranzo et al., 1999; Meara et al., 1999).

TNP has been described as an adjunctive treatment of sternal wound infections (Hersh et al., 2001), poststernotomy mediastinitis (Obdeijn et al., 1999) and chest wall dehiscence (De Lange et al., 2000) following cardiac surgery. In these three reports, TNP therapy was believed to create a clean, well vascularised and more 'stable' smaller wound defect, promoting granulation tissue development and avoiding the need for repeated debridement procedures or further surgery.

TNP has been used recently to obtain serial closure of dehiscenced or infected abdominoplasties in four cases averting the need for corrective surgery (Fenn and Butler, 2001).

1.1.2.2 Wounds with exposed bone or implants

The use of TNP has been described to increase granulation tissue formation over exposed bone and metal implants (Argenta and Morykwas, 1997a; Fleischmann et al., 1995; Müllner et al., 1997). Recently, Defranzo et al. (2001) have published

their updated series of 75 patients with lower extremity wounds with exposed bone that underwent TNP therapy at 125mmHg and reported successful coverage without complications in 71 of these cases. TNP was applied directly over exposed bone without periosteum but did not give details of how large the defects were, but acknowledged that large exposed plates or large areas of exposed bone would not be good candidates for TNP. They suggested that granulation tissue developed from surrounding viable soft tissue or possibly from elements beneath the fracture site (DeFranzo et al., 2001).

1.1.2.3 Chronic wounds

The application of TNP therapy to chronic wounds has been extensively described since the early work of Argenta (Argenta and Morykwas, 1997a; Deva et al., 2000; Müllner et al., 1997). Argenta and Morykwas (1997) treated one hundred and seventy-five pressure, venous stasis, radiation and diabetic ulcers, and other miscellaneous chronic wounds using the V.A.C. system with a mixture of continuous or intermittent subatmospheric pressure. They showed that the "...vast majority of chronic wounds (171 of 175) responded favourably" to treatment, noting that there appeared to be significant removal of oedema and proliferation of granulation tissue in their treated cases. The treatment lengths varied between patients and successful outcomes were gauged subjectively. Further definitive treatments (skin grafting, flap closure or healing by secondary intention with dressings) were based on clinical judgement. They did not measure the observed attenuation of oedema but stated that the surrounding tissues became 'more pliable' and they did not measure the rate of increased granulation tissue formation either, in these cases. They stressed in their report that many of these chronic wounds were recalcitrant to past surgical or dressing procedures (although they did not specify exactly how many), or were considered non-surgical candidates because of concomitant disease (Argenta and Morykwas, 1997a).

1.1.2.4 Burn wounds

Despite suggestions of its potential use in burn wounds (Morykwas et al., 1999a; Morykwas and Argenta, 1997), there are no published accounts of TNP therapy in the treatment of human burns.

1.1.2.5 Miscellaneous uses of TNP

A number of authors have used TNP as a dressing for skin graft fixation to aid contouring the graft to irregular parts of the body (Banwell et al., 1999; Blackburn et al., 1998; Schneider et al., 1998). These studies have suggested that the advantage of TNP as a method of skin graft fixation may be due to its ability to remove excess tissue fluid and haematoma formation from beneath the graft and providing secure immobilisation at the wound-graft interface. Despite this, there have been no comparative clinically controlled trials published testing this form of fixation with standard skin graft bolster techniques.

Greer et al. (1999) have used TNP to stimulate granulation tissue coverage of exposed tendon (without paratenon) to the radial forearm free flap donor site where skin graft cover had broken down in two cases (Greer et al., 1999b).

TNP has also been used in the sealing of lymphocutaneous fistulas (Greer et al., 2000), suggesting that granulation tissue formation was likely to be the reason for its success in the treatment of the two cases they describe.

1.1.2.6 Clinically controlled trials involving the use of TNP on wounds

Recently, there has been an attempt to focus work on clinically controlled trials to evaluate TNP therapy in wound healing. There are currently only three clinically controlled trials published to date, assessing the efficacy of TNP therapy in a clinical setting (Genecov et al., 1998; Joseph et al., 2000; McCallon et al., 2001) and one preliminary trial in abstract (Greer et al., 1999a).

One trial involved the use of TNP on skin graft donor sites to assess rates of reepithelization by histology, against an intra-patient controlled Opsite™ dressing (Genecov et al., 1998). Details of this work have been described in section 1.1.2.1 on page 21.

Two trials have been published to compare TNP therapy with saline-moistened gauze dressings in chronic wounds (Joseph et al., 2000; McCallon et al., 2001). Both of these have been subject to an extensive review by the Cochrane Wounds Group, whose remit was to determine the efficacy of TNP therapy in chronic wounds by tracing all available randomised controlled trials (Evans and Land,

2001). The two randomised controlled trials reviewed above were conducted on a small number of patients and objective outcome measures were difficult to define and record. Despite these problems, it was recommended that there is only weak evidence to suggest that TNP may be superior to saline-gauze dressings in the treatment of chronic wounds (Evans and Land, 2001).

Recently, Fleischmann and colleagues have conducted a randomised controlled trial to determine the influence of TNP on the systemic inflammatory response (Buttenschoen et al., 2001). Thirty-five patients who required open reduction and fixation of isolated closed ankle fractures (within 6 hours of injury) were randomised to direct surgical closure (n=18) or TNP using V.A.C. dressings with PVA foam at subatmospheric pressures of 600mmHg for up to 120 hours (n=17). They measured a number of inflammatory acute phase reactants (including endotoxin, CRP, IL6, α_1 -protease inhibitor and complement) in plasma before and at 0.5, 12, 24, 48 and 96 hours after surgery. They reported peak endotoxin levels at 0.5 hours after surgery in the skin-sutured group (0.11 EU/ml), which was absent in the TNP group (0.07 EU/ml) with pre-op values at 0.06 EU/ml. This difference between the two groups was insignificant at all other times. The other inflammatory parameters measured in plasma were not significantly different. They suggested that TNP therapy does not alter the systemic inflammatory response (Buttenschoen et al., 2001). No attempt was made to determine whether TNP might change the local environment of the wound.

1.1.3 Evidence for Mechanisms of Action of TNP in Wound Healing

A number of factors have been proposed as mechanisms that might explain the observed clinical effects of TNP. The evidence suggesting a putative role for each of these factors is reviewed in detail below and, where possible, relates to studies using TNP foam dressings, unless stated otherwise.

1.1.3.1 Enhanced microvascular blood flow

Using TNP foam dressings on full thickness wounds in pigs, Morykwas et al. (1997) have shown that blood flow, measured using Laser Doppler needle probes both within the wound and adjacent to it, recorded peak blood flow of four times baseline values at subatmospheric pressures of 125mmHg. Furthermore, they observed that at constant negative pressures of 125mmHg, the local blood flow declined after 5 to 7 minutes to a baseline reading. These flow measurements were also immediately depressed below baseline levels at levels of 400mmHg or more (Morykwas et al., 1997). Using the results of this work, these authors determined an optimal intermittent TNP cycling regimen of 5-minutes-on and 2-minutes-off. They postulated that the increase in blood flow measured in their experimental cases might be due to active removal of interstitial fluid from the tissues surrounding the wound, which decompress small blood vessels and restoring blood flow. They did not provide any evidence to support this view.

Morykwas and colleagues also created 3x12cm random pattern flaps on the backs of pig, which were raised and resutured to the skin. Flaps were assigned to one of four groups: (1) flap exposed to TNP both prior to (4 days) and following surgery; (2) flap exposed to TNP prior to surgery but not post surgery; (3) flap exposed to TNP post surgery only; (4) flap that received no TNP. At 72 hours, photographs were taken and acetate drawings made to determine % flap survival based on areas of discolouration. Their results showed that pre- and post-treated flap survival (72.2% +/-10%) was significantly greater than the control group (51.2% +/-6.9%). No significant differences were shown between pre- and post- treated flaps. The authors suggested that nutrient blood flow might be enhanced using TNP as determined by the survival of these random-pattern flap experiments (Morykwas et al., 1997).

1.1.3.2 Stimulation of granulation tissue formation

Using circular 2.5cm full thickness skin wound models in the pig, Morykwas et al. (1997) measured the defect space daily by taking alginate impressions. The wounds were created on the dorsal midline, which they claimed did not undergo contraction during healing. Rates of granulation tissue formation measured by the volume displacement of the casts showed that the TNP treated wounds increased

63.3% (SD±26.1%) and 103.4% (SD±35.3%) in continuous and intermittent TNP-treated groups respectively, as compared to saline-soaked gauze controls. They argued that the diameter of the wounds did not change during the course of the study and that any differences in volume were accounted for by increases in granulation tissue alone (Morykwas et al., 1997). More recently, Morykwas et al. (2001) showed that acute dermal pig wounds treated with TNP, granulated to a level equal to surrounding tissue by 8 days with subatmospheric pressures of 125mmHg with only 21.2% granulation tissue filling with 25mmHg and 5.9% granulation tissue filling with 500mmHg. Based on this in vivo animal evidence, they proposed that 125mmHg was the optimal subatmospheric pressure in TNP therapy (Morykwas et al., 2001).

Fabian et al. (2000), using a skin-excised rabbit ear wound model, have demonstrated significantly greater granulation tissue formation (measured directly using a lens micrometer) on TNP-treated experimentally-induced ischaemic wounds than a control wound of foam but without suction at 10 days post injury (Fabian et al., 2000). The histological features of granulation tissue are dominated by the proliferation of new small blood vessels, fibroblasts and macrophages (Kumar et al., 1992). From the literature review, no studies have looked directly at the effects of TNP on these factors directly.

1.1.3.3 Reduction in bacterial colonisation

Experimental wounds in pigs inoculated with a human isolate of *Staphylococcus aureus* (n=3) and a pig isolate of *Staphylococcus epidermidis* (n=2) were subjected to TNP at a constant 125mmHg and compared to a control group on the same animal (Morykwas et al., 1997). Daily biopsies of the wounds were taken for up to 2 weeks. The tissue collected was homogenised, diluted in sterile saline and incubated on the surface of a blood agar plate. Colonies were counted and the number of organisms per gram of tissue calculated. The results showed significantly reduced organisms, falling from an inoculum of 10^8 organisms per gram of tissue to less than 10^5 organisms per gram of tissue (the traditionally accepted upper level for infection) between day 4 and 5 only in the TNP-treated group as compared to controls. The control group did not fall to below 10^5 until after a mean of 11 days (Morykwas et al., 1997). This work mirrors some reports using TNP therapy in the clinical setting, which have noted a reduction in

superficial purulence and odour (concomitant with the presence of bacteria and leucocytes) in the effluent that contained quantitatively reduced bacterial counts over a similar time of 3-4 days although no details were given (Argenta and Morykwas, 1997a). It remains unclear whether the reduced bacterial counts noted in the above studies are due to the direct removal of bacteria or secondary to enhanced phagocytosis due to the increase in local blood supply or some other mechanism.

1.1.3.4 Changes to interstitial fluid volume

Some authors believe that the increase in local blood flow following TNP therapy is due to the active removal of excess interstitial fluid volume from the wound and its surroundings which might reduce the tissue pressure around the skin microcirculation and improve the local blood flow (Argenta and Morykwas, 1997a; Cozart et al., 1999). Despite the measurement of large amounts of effluent from the drains of patients treated with TNP therapy in clinical series, there are no reports in the literature providing direct objective human or animal experimental evidence that measure alterations in interstitial fluid volume from wounds under the influence of TNP therapy.

1.1.3.5 Removal of factors within wound exudates

Elevated levels of proteolytic enzymes present in chronic wound fluids (Bucalo et al., 1993; Grinnell et al., 1992; Trengove et al., 1999) and burn wound fluids (Grinnell and Zhu, 1994; He et al., 1998; Neely et al., 1997) are hypothesized to impede normal wound healing (Section 1.4.3 on page 63). Despite the suggestion that TNP therapy might alter the composition of wound fluid (Argenta and Morykwas, 1997a; Banwell, 1999), there is no evidence from the literature that the use of TNP therapy actually does so, and no comparisons have been reported between wound fluids retrieved from TNP-treated wounds and control wounds within the same patient. Furthermore, this literature review has been unable to trace any published study that has used TNP to collect and analyse the fluid composition of TNP-treated wounds in an experimental or clinical setting, to link the efficacy of TNP to alterations in the constituents of wound fluid.

One paper by Morykwas et al. (1999) has examined the effects of TNP therapy on Doxorubicin-injected pig skin (Morykwas et al., 1999b). Doxorubicin was injected intradermally through intact skin to simulate an extravasation incident. Following this, the epidermis was perforated by two holes created with a 2mm punch biopsy to allow a path for egress of the injected Doxorubicin solution, although no tissue was removed. In a TNP-treated group (n=16), no extravasation-induced ulcers were produced, compared to a control group (n=16) in which 10 ulcers developed. They explained that this was most likely due to ‘...Doxorubicin molecules migrating out of the underlying tissues through the epidermis into the vacuum dressing...’ and that the prevention of extravasation ulcers might be due to an enhanced diffusion gradient formed by the suction. They did not measure the volume of aspirate from either the TNP-treated or control sites, nor did they attempt to detect Doxorubicin directly from the aspirates. They claimed that the egress of the agent was not facilitated by the punch biopsy holes created in the skin, but instead directly removed through an intact stratum corneum. Unfortunately, it was not made clear in this paper whether extravasation ulcers that formed were directly associated with the perforated punch biopsy sites in the epidermis (Morykwas et al., 1999b).

It remains a matter of conjecture whether TNP therapy can directly influence the removal of wound exudates through open, injured skin or extravasated chemotherapeutic agents through intact skin.

1.1.3.6 Response of tissues to mechanical forces

It has been proposed that TNP therapy may exert a mechanical effect on wounds at both a ‘macroscopic’ (directly mechanical) and a ‘microscopic’ (cellular and/or molecular) level (Banwell, 1999). It is difficult to demonstrate a precise mechanical effect of TNP on tissues, cells or their humoral effectors (e.g. growth factors or proteases), because of the complexities in methodological design. For example, section 1.1.3.2 describes a study in which Morykwas et al. (1997) evaluated the effects of TNP on granulation tissue formation by measuring the decrease in wound size using alginate casts on the dorsum of pigs (Morykwas et al., 1997). They claimed that the observed decreases in wound size were not due to wound contraction caused by direct mechanical effects of TNP but instead, due

to real increases in granulation tissue filling the wound. They postulated that the increases in granulation tissue were due to mechanical forces imposed on surrounding tissues through viscoelastic flow or directly on cellular proliferation (presumably of endothelial cells and of fibroblasts into myofibroblasts).

It is not clear whether these observations noted are due to: (1) direct mechanical forces on tissues, (2) mechanical influences applied to cells or their effector functions or (3) indirect, non-mechanical influences, such as the reduction in oedema or enhanced blood flow.

In an effort to define the role of mechanical stress on skin directly, the influence of tissue expansion techniques in normal skin of animals and humans has been studied supporting the notion that tissue expansion increases mitotic activity in skin to generate a net gain in tissue (Austad et al., 1986; Olenius et al., 1993). TNP therapy has been described as a form of 'reverse tissue expansion' with the implication that any mechanical effects of TNP therapy might be likened to those found using tissue expansion techniques (Banwell, 1999). However, caution is required in comparing putative mechanical effects of TNP with studies involving tissue expansion for two principle reasons. Firstly, the viscoelastic effects on skin by tissue expansion and TNP are different. In mechanical terms, tissue expansion causes the skin to stretch to a specific length until a force is generated and then fixed at that position. The force generated then diminishes as relaxation occurs. Gibson described this as stress relaxation: a constant elongation with diminishing load (Gibson, 1977). Furthermore, by its intermittent nature, tissue expansion is best thought of as 'stepped stress relaxation' (Brody, 1993). Meanwhile, TNP therapy is an example of mechanical creep (Gibson, 1977): an elongation secondary to a constant force. As the surrounding skin is subjected to a constant force under the TNP dressing, the skin is dragged inwards to affect closure, as noted clinically in the treatment of chronic ulcers for example. Secondly, Austad et al. (1982) described tissue expansion as 'relatively benign'. Conducting controlled tissue expansion studies on Guinea Pigs, they found no evidence of necrosis or inflammation histologically, during a 1 to 18 week period (Austad et al., 1982). In contrast, TNP therapy is applied to acute or chronic inflamed wounds and circumstantial comparisons to tissue expansion studies performed in normal (non-inflamed) skin using foam dressings cannot be safely made.

Mechanical (suction) forces directly influence angiogenesis (Ryan and Barnhill, 1983). In vitro studies have shown that tritiated thymidine-incubated vascular cells (Sumpio et al., 1987) and smooth muscle cells (Wilson et al., 1993) demonstrate statistically increased cell proliferation and DNA synthesis in vacuum operated flexible-bottomed culture plates compared to controls; and blood vessel density is also increased following mechanical strain experiments in vitro (Baker and Sanders, 2000).

The mechanism(s) by which cells 'sense' or respond to mechanical stimuli is not clear. Little is known about the underlying molecular pathways by which mechanical events are transduced into intracellular physiological responses and this area is beyond the scope of this review. However, the importance of the ECM in mediating mechanical forces has been shown by observations that strain-induced cell proliferation is matrix-dependant. For example, strain plates were coated with a number of ECM proteins (including fibronectin, vitronectin and collagen) to show matrix-induced differences in vascular smooth muscle cell proliferation under cyclic strain. Mechanical strain was applied by cyclical application of a vacuum (up to 150mmHg) under the plates, to show DNA-labelled cells adhering, spreading and proliferating on ECM proteins (Wilson et al., 1995).

The exact nature of the biomechanical effects of TNP on skin is not known. There have been no published studies to specifically evaluate the different mechanical effects of TNP therapy (shear stress resulting from fluid flow, direct compression or tensile stress) on normal skin or wound histomorphology or the type of mechanical loading (incremental, constant or cyclical). Instead, circumstantial evidence suggests that at a macroscopic tissue level, centripetal forces may act to draw skin edges together under the influence of mechanical creep. Equally, mechanical forces may be sensed intracellularly via the ECM or cell membrane directly to alter cell growth and proliferation.

1.1.4 Topical Negative Pressure Therapy in Human and Animal Burn Wounds

The relationship of blood flow patency to burn wound survival and the possibility that the ischaemia (and subsequent necrosis) observed in deep partial thickness

burns might be prevented from progressing deeper, has led to the suggestion that TNP therapy may provide a useful means of treatment of partial thickness burn injury (Morykwas et al., 1999a); see section 1.2.3.

Morykwas et al. (1999a) conducted a series of experiments to determine whether TNP therapy might prevent progressive partial thickness burn injury. Partial thickness burns were created on porcine skin, and continuous TNP therapy, at pressures of 125mmHg, commenced either immediately or at delayed intervals of 6, 12, 18, or 24 hours from the start of injury up to 48 hours later. They wanted to establish the maximal interval between burn injury and the application of TNP, which might prevent burn progression. A further group of pigs were burnt on both the right and left side in the same manner as before, but underwent TNP therapy for 6 hours and 12 hours respectively. This was performed to determine the minimum duration of TNP to prevent burn progression. On a daily basis, paired biopsies of control and TNP-treated partial-thickness burns were excised, paraffin-fixed and trichrome-stained for measurements of maximum depth of 'cellular' death using collagen denaturation as a histological marker.

From the histological results of their study, they suggested that TNP-treated burns 'totally re-epithelized by day 5' regardless of the onset of TNP therapy or its duration, whereas control wounds were only partially epithelialised, completely reepithelialising by day 9.

Using depth measurements of collagen denaturation with the trichrome stain (which they described as a dark band of tissue), they determined that the maximum depths of cellular death for TNP-treated burns were 0.095, 0.275, 0.613 and 0.738mm at 0, 12, 18 and 24 hours delayed treatment. The control wounds measured 0.885mm. The depth of cellular death for the 0 and 12 hour delayed wounds were significantly less than for the 18, 24, or control wounds. These authors emphasised the importance of oedema as a critical factor in compromising dermal microcirculation, and suggested that by reducing the amount of denatured collagen in the TNP-treated wounds, this might reduce the oedema present in the skin, the removal of which would result in an increase in local perfusion. They did not provide any evidence to support this hypothesis. Furthermore, they suggested that colour changes in trichrome staining might be used as a marker of heat-denatured collagen (Chvapil et al., 1984), but the thermal energy necessary to denature collagen far exceeds that which may cause vascular occlusion or

irreparable cellular damage (Watts, 1998), so caution is required in using this technique for burn depth assessment.

They also claimed that the number of inflammatory cells in the TNP-treated burn group was less, although they did not mention the types of inflammatory cells observed or provide evidence of this (Morykwas et al., 1999a).

Banwell et al. (2000) randomised 32 partial-thickness skin burn experiments on pigs for TNP therapy or foam dressings alone. Microvascular blood flow dynamics were determined using laser Doppler scanning and dermal viability recorded by the number of tangential burn excisions required to reach healthy bleeding dermis. They showed significant increases in flux values (a measure of perfusion through the microcirculation) using the laser Doppler scanner at 48 and 72 hours compared to controls. The average number of burn wound excisions in the TNP-treated group (6.25 excisions) was also significantly reduced compared to the controls (14.9 excisions). They concluded from this work that TNP might reduce dermal ischaemia and preserve dermis in partial thickness burn wounds. Unfortunately, they did not support these findings with histological correlates (Banwell et al., 2000).

No published studies have reported on the efficacy of TNP therapy in the treatment of partial-thickness burns in humans.

1.2 Pathophysiology of Cutaneous Wound Healing: The Role of Neutrophils

1.2.1 Wound repair

Wound healing is a highly regulated and complex interaction involving cells, extracellular matrix and soluble mediators, including growth factors and cytokines (Tarnuzzer and Schultz, 1996). Three overlapping phases of wound repair exist: (1) the inflammatory phase; (2) the proliferative phase; and (3) the maturation/remodelling phase. Problematic wound healing can occur following an imbalance between cellular proliferation and local degradative processes, which can lead to either excessive scarring (collagen deposition) or a delay in wound healing (due to extracellular matrix degradation) (Clark, 1996).

1.2.2 Neutrophils in wound repair

The non-specific inflammatory phase of wounding is characterised by the activation and recruitment of neutrophils followed later by monocytes. Neutrophils are the most common of all the circulating leucocytes (70%). Following injury, neutrophil numbers peak at 24 hours and then gradually fall in inflamed tissues (Slavin, 1996). Under normal circumstances, neutrophils disappear from the wound by 3 days (Mast and Schultz, 1996), most likely by apoptosis (Greenhalgh, 1998). Neutrophil activation and recruitment is mediated by a large number of vasoactive mediators and chemotactic factors generated from the activation of the coagulation, complement and arachidonic acid pathways, and from injured or necrotic parenchymal cells within the damaged tissues (Clark, 1996).

1.2.2.1 Complement components and related factors

Complement products induce a range of neutrophil activation events, including polarization, chemotaxis, oxygen-free radical production, degranulation and upregulation of neutrophil adhesion molecules (Khan et al., 1996). Injured tissue activates the complement cascade and becomes coated in fragments of C3. The

role of C3b, in particular, is to facilitate attachment of neutrophils to objects coated with them. Neutrophils express complement receptors CR1 and CR3 (CD11/CD18), which are required for opsonization of bacteria and cellular debris. C3a and C5a expression from the complement cascade are potent chemotactic agents for neutrophils into sites of tissue injury and upregulates the expression of neutrophil surface receptors, including CR1 and CR3. Increased levels of these anaphylatoxins are considered to regulate neutrophil function in the plasma of burned patients (Arturson, 1995).

1.2.2.2 Cytokines

Cytokines (including growth factors and interleukins) are soluble peptides, which mediate inflammatory events at low concentrations. A range of cell types produce cytokines, which behave in a complex autocrine and paracrine fashion to activate endothelial cells and leucocytes (Ravage et al., 1998), including neutrophils (Piccolo et al., 1999). The effects of individual cytokines are difficult to determine since multiple interactions between them affect their synthesis and clinical effects in diverse ways (Cohen and Cohen, 1996).

Following any skin injury, the inflammatory phase is initiated by the release of PDGF, IGF-1, EGF and TGF- β from platelet degranulation. TNF- α produced by damaged endothelial cells, keratinocytes and fibroblasts chemotactically draw inflammatory cells in to the wound (Mast and Schultz, 1996). Newly arriving neutrophils release additional growth factors following activation (Tarnuzzer and Schultz, 1996). Levels of active cytokines have been measured in both burn wound fluid (Ono et al., 1995b), donor site wound fluid (Ono et al., 1995a) and other surgical wound fluids (Tarnuzzer and Schultz, 1996). By direct sampling of wound fluid over time within the same wound using advances in assay technology, it is possible to measure the patterns of cytokine expression, which have provided insights into the roles these mediators play during the course of both normal (Dvornich et al., 1992) and poorly-healing wounds (Cooper et al., 1994). Neutrophils also secrete further pro-inflammatory cytokines (TNF- α and interleukins) to recruit more neutrophils and macrophages (Dubravec et al., 1990; Mast and Schultz, 1996).

1.2.2.3 Arachidonic Acid Metabolites

Arachidonic acid (AA) is released from cell membranes following injury. The metabolism of AA results in prostaglandin production via the cyclo-oxygenase pathway or leukotrienes via the lipoxygenase pathway. These products activate neutrophils. A massive release of prostanoids occurs after burns, the commonest are prostacyclin (PGI₂) and thromboxane A₂ (TxA₂). TxA₂ is thought to be responsible for increase platelet aggregation and neutrophil margination and may be responsible for the decrease in blood flow in burn tissue. PGI₂ is a potent vasodilator and accentuates the vascular permeability. Leukotrienes are produced by neutrophils, macrophages and vascular tissue and some are potent neutrophil chemoattractants. Their current role in burn injury is unclear (Youn et al., 1992), but may be involved in neutrophil adhesion or activation of Reactive Oxygen Species (ROS) and proteolytic enzymes (Smedly et al., 1986).

The accumulation of leucocytes in inflamed tissue results from adhesive interactions between leucocytes and endothelial cells within the postcapillary venules of the microcirculation. An orderly sequence of events underlies the cell-cell interactions, which are mediated by specific adhesion molecules, and involves neutrophil rolling, adherence, and emigration (Granger and Kubes, 1994). These important processes have been uncovered by the use of in vitro binding assays of leucocytes to endothelial monolayers (using immunoneutralisation MoAbs), and in vivo using leucocyte-endothelial cell adhesion models (intravital videomicroscopy) and 'knock-out' mice (Panes et al., 1999).

1.2.3 Neutrophils in the pathophysiology of thermal injury

Burns are classified according to the depth of tissue damaged (British Burns Association, 1996). In practice, burns usually exhibit areas of mixed depth. Epidermal burns are the most superficial burn and involve the epidermis alone. Healing occurs quickly within 7 days without treatment. Superficial partial thickness burns include the epidermis and upper (papillary) dermal layers of the skin. The blister is the hallmark of this burn. These burns heal with conservative treatment within 14 days from epidermal remnants within the base of sweat ducts

and hair follicles. Scarring is minimal. Deep partial thickness burns cause damage deeper into the reticular dermis. The clinical appearance of the remaining dermis is a blotchy red colour (fixed capillary staining), due to extravasation of haemoglobin from red blood cells leaking from damaged vessels. Pinprick sensation is reduced since a greater proportion of sensory nerves are destroyed at this deeper level of skin injury. Healing takes longer than 2 to 3 weeks and is characterised by residual scarring. Full thickness burns damage the entire thickness of the skin and may penetrate more deeply into underlying structures. Due to the loss of epithelial elements, the nature of healing can only occur by wound contraction and epithelial cell migration. Surgery is usually mandatory.

Jackson (1953) described patterns of dermal blood flow in partial thickness burn wounds that mirror the clinical depths of burn injury. He described a burn wound as consisting of three concentric zones of decreasing degree of cellular injury: a zone of coagulation, a zone of stasis and a zone of hyperaemia (Jackson, 1953). He identified the middle 'zone of stasis' burns microscopically, observing that the capillaries were dilated and packed with red blood cells by four hours. He described a cessation of the circulation within the dermis, as the zone became progressively ischaemic and then necrotic over a period of 24 – 48 hours following burn injury (Jackson, 1953). This phenomenon of dermal ischaemia seen in some partial thickness burns, which become deeper over this time, has been noted clinically (Godina et al., 1977). Histologically, this secondary tissue loss is seen as necrosis within the zone of stasis, and occurs as a sequel to progressive vascular occlusion (Smahel, 1991). Zawacki et al. (1974) described how the progressive injury in the zone of stasis was reversible with the prevention of wound dehydration (Zawacki, 1974).

Brånemark (1968) described the appearance of granulocytes during microvascular stasis in human burns as '...often rigid and may block the nutritive capillaries temporarily or even permanently.' when observing these under light microscopy. He also observed the '...disruption of granulocytes with liberation of their granules into the lumen...' which he suggested was significant in the development of tissue injury (Brånemark et al., 1968).

Boykin et al. (1980) studied the cutaneous microcirculation dynamically in the ear of a hairless mouse model following an experimental scald. He noted a zone of complete capillary occlusion, then partial zone of occlusion and then zone of

hyperaemia in these experimental cases that corresponded to Jackson's original descriptions. Furthermore, he also reported a 10-fold progression of complete capillary occlusion during the following 48 hours postburn. He observed sticking of leucocytes to the endothelium of post capillary venules causing partial occlusion of the vessels between 8 and 24 hours, although he never observed 'leucocyte sticking' as causing complete vascular occlusion. He suggested that the cause of this capillary occlusion was a combination of red cell aggregates, leucocyte adhesion to venular walls and platelet thromboembolism (Boykin et al., 1980).

Tyler (1998) has observed neutrophil extravasation into surrounding tissues in partial thickness burns, which was apparent within 6 hours of burning and reported that neutrophil counts correlated directly with burn depth at 48 hours (Tyler, 1998).

1.2.4 Evidence for neutrophils as mediators of burn depth progression

Experiments that have excluded neutrophils from the burn environment by manipulation of the adhesion molecules that control their vascular transmigration have provided insights into the role of neutrophils in progressive dermal injury.

Mileski et al. (1992) investigated the role of neutrophils in progressive microvascular injury following burns to rabbit skin. They used systemic monoclonal antibodies directed against the CD18 part of the adhesion complex (MoAb R15.7), and its endothelial ligand ICAM-1 (MoAb R6.5) given 30 minutes postburn. Using laser Doppler blood flow as an assessment of microcirculation, they demonstrated that both R15.7 and R6.5 resulted in improved microvascular blood flow during the following 72 hours. They concluded that blocking neutrophil adhesion to the endothelium with antiCD18 and anti-ICAM-1 improved microvascular perfusion in the marginal zone of stasis following thermal injury in this model (Mileski et al., 1992).

Bucky et al. (1994) used a MoAb (60.3) directed to CD18 in a similar rabbit model, 30 minutes following partial-thickness burns. They reported a reduction in burn surface area at 24 hours, and higher hair follicle counts and reepithelialisation at 8 days compared to controls. They concluded that neutrophil adherence played an important role in progressive tissue destruction within 'zone of stasis' burns (Bucky et al., 1994).

Choi et al. (1995) administered a MoAb (M2) directed against CD11b following a brass comb burn that create unburned interspaces as a model of the zone of stasis, to monitor microvascular blood flow. Prevention of loss of dermal vascular flow in the interspaces was demonstrated by laser Doppler monitoring. Latex vascular casts revealed patency of vessels within the comb interspaces compared to controls. Histologically, neutrophil extravasation was blocked in the interspaces using this MoAb, with intraluminal and perivascular neutrophil counts significantly greater in the control groups. They concluded that by reducing or eliminating the infiltration of neutrophils into dermal tissues adjacent to a burn prevented delayed secondary necrosis (Choi et al., 1995).

Mileski and colleagues (1996) repeated some of their experimental work in rabbits using MoAbs to L-selectin (Dreg 200) and CD18 (R15.7). The degree of neutrophil infiltration was recorded after immunohistochemical staining and matched to dermal blood flow assessed by laser Doppler. They showed that neutrophil accumulation was partially L-selectin dependent, but wound perfusion and tissue necrosis were unaffected. This contrasted with CD18, which improved perfusion and reduced tissue necrosis, but did not affect the degree of neutrophil infiltration when compared to controls (Nwariaku et al., 1996).

Similar neutrophil-mediated tissue injury has also been demonstrated in skin flaps (Cetinkale et al., 1998) and ischaemic-reperfusion injury (Hernandez et al., 1987) and this damage has been prevented by a number of different anti-neutrophil agents including monoclonal antibodies (Hernandez et al., 1987) and immunosuppressive agents such as cyclosporin (CyA) and FK506 (Cetinkale et al., 1997; Cetinkale et al., 1998).

1.2.5 Mechanisms of Neutrophil-mediated injury

Neutrophil-mediated tissue injury following the activation of neutrophils at sites of inflammation has been implicated in a number of inflammatory diseases (Anderson et al., 1991). Neutrophils produce over 50 toxins, mainly reactive oxygen species (ROS) and proteases (Henson and Johnston, 1987).

1.2.5.1 Reactive oxygen species

Free radicals are generated deliberately by neutrophils (Paty et al., 1990) to catalyse intracellular reactions and during the 'respiratory burst' in which oxygen is consumed during phagocytosis by activated cells, as part of their bactericidal role (Latha and Babu, 2001). However, the balance between normal free radical production and their endogenous scavengers can be lost; the local production of ROS is a common factor responsible for secondary tissue damage in ischaemic-reperfusion (IR) injuries (Welbourn et al., 1991) and burns (Latha and Babu, 2001), with the neutrophil implicated as the primary source of ROS (Weiss, 1989). Cellular damage by ROS includes lipid peroxidation of cell membranes, disruption of intracellular organelle membranes, direct endothelial damage with increasing vascular permeability and inactivation of anti-proteases, which may lead to unopposed protease activity with increased cellular and ECM destruction (Kaufman et al., 1989; Latha and Babu, 2001).

A number of investigators have provided evidence that ROS is implicated in the pathogenesis of progressive ischaemia in the zone of stasis in animal models (Cetinkale et al., 1997; Cetinkale et al., 1999; Choi and Ehrlich, 1993), although others have reported no significant effects (Dyess et al., 2000; Melikian et al., 1987).

Despite the role of neutrophil-derived ROS in cellular and tissue injury, these agents alone are not capable of reproducing all the events associated with tissue destruction and cooperation between proteases may enhance its effects synergistically (Ginsburg and Kohen, 1995). Indeed, Weiss (1989) forwarded the hypothesis that synergy might exist between proteases and ROS in neutrophil-mediated tissue injury. He described neutrophil-derived elastase in tissue destruction of connective tissue matrix and its activity, which could be greatly facilitated if its endogenous inhibitor α_1 -protease inhibitor (α_1 -PI) were destroyed. The hypohalous acid, HOCl, generated by the PMN myeloperoxidase-H₂O₂-halide system could destroy these inhibitors, thereby augmenting the effect of the protease on host tissues (Weiss, 1989).

Ginsburg and Kohen (1995) have reviewed these concepts and have proposed that matrix proteolysis correlates more closely to the release of proteases from neutrophils than with the generation of ROS (Ginsburg and Kohen, 1995).

1.2.5.2 Neutrophil Proteases

These proteases have the capability to degrade key components of the extracellular matrix (Weiss, 1989) and have been implicated in neutrophil-mediated endothelial cell injury (Ward and Varani, 1993). A review of leucocyte-derived proteases is discussed in detail in section 1.3 on page 42.

1.3 Neutrophil Elastase and its Inhibitors in Wound Healing

1.3.1 Classification of Leucocyte Proteases

Four distinct classes of leucocyte proteases have been identified by the biochemical mechanisms responsible for their catalytic activity.

1.3.1.1 Serine Proteases

Serine proteases are the largest class of mammalian proteases. Their activity is dependent on a catalytic triad consisting of residues Asp¹⁰², His⁵⁷, Ser¹⁹⁵ (Owen and Campbell, 1999). This family includes pancreatic trypsin, chymotrypsin, kallikrein, coagulation and complement proteases (plasmin, thrombin, PA), Neutrophil Elastase (NE), Cathepsin G (CG) and Proteinase 3 (PR3). Some serine proteases are synthesised as inactive precursors that require limited proteolysis to activate them. However, NE, CG and PR3 are notable exceptions because they are stored in an active form within neutrophil azurophilic granules (see Table 2 on page 45).

The importance of serine proteases in physiological and pathological processes is underscored by the fact that the inhibitors of these enzymes (Serpins) comprise about 10% of all plasma proteins. The major class of Serpins (α_1 -protease inhibitor, α_1 -antichymotrypsin, α_2 -plasmin inhibitor, antithrombin III, plasminogen activator inhibitors, C1 inhibitor) are synthesised and secreted by hepatocytes, along with the universal inhibitor, α_2 -macroglobulin (Owen and Campbell, 1999).

1.3.1.2 Metalloproteinases

Metalloproteinases (MMPs) are dependent on intrinsic zinc ions for their catalytic activity. At least 20 different MMPs have been characterised (Parks, 1999); 9 of these are known to be expressed by leucocytes, and two (MMP-8 and MMP-9) are released from neutrophils. MMP-8 is capable of degrading collagens I, II, III, VII, X, gelatin, proteoglycans, bradykinin and substance P, and can degrade α_1 -protease inhibitor. MMP-9 degrades gelatin, collagen IV, V, VII, X, XI, fibronectin, laminin and elastin. The majority of MMPs are not secreted from cells in an active

form but require cleavage of their pro-forms (zymogens) into active enzymes. Latency of MMPs is maintained by the linkage between cysteine in the proenzyme domain and zinc at the catalytic site (Parks, 1999).

Regulation of MMP activity occurs at the level of: (1) gene transcription, (2) activation of pro-forms, and (3) inhibition by inhibitors. MMPs are specifically inhibited by the naturally occurring tissue inhibitors of metalloproteinase (TIMPs), which are synthesised by connective tissue cells and leucocytes and form non-covalent complexes with MMPs. Four members of the TIMP family have been identified (TIMP-1, -2, -3, and -4) (Owen and Campbell, 1999).

1.3.1.3 Cysteine Proteases

Four human lysosomal proteases (Cathepsin B, H, L and S) are synthesised as proenzymes and are activated within lysosomes. Functioning at acidic pH, these enzymes play important roles in intracellular protein digestion, although they have been implicated in extracellular proteolytic events (Owen and Campbell, 1999). This class of leucocyte protease will not be discussed further.

1.3.1.4 Aspartic Proteases

Examples of aspartic proteases include Cathepsin D and E. Two aspartic acid residues are integral to their catalytic activities, which are functional at acidic pH. Cathepsin D has been shown to degrade proteoglycans extracellularly and is released by macrophages during the inflammatory response. This class of leucocyte protease will not be discussed further.

Table 1 Classification of Leucocyte Proteases

Mechanism	pH optimum	Examples	Location
Serine Proteases	Neutral (pH 7-9)	Neutrophil Elastase	PMN, monocytes
		Cathepsin G	PMN, monocytes
		Protease 3	PMN, monocytes
		uPA	PMN, monocytes, macrophages
		Tryptase	Mast cells, basophils
		Chymase	Mast cells
Metalloproteinases	Neutral (pH 7-9)	MMP-1	Mononuclear phagocytes
		MMP-8	PMN, eosinophils
		MMP-2	Mononuclear phagocytes, fibroblasts
		MMP-9	PMN, mononuclear phagocytes
		MMP-3, -10, -11	Mononuclear phagocytes
		MMP-7	Monocytes
		MMP-12	Macrophages
Cysteine Proteases	Acidic (pH 3-6)	Cathepsin S	Lysosomes of most cells
		Cathepsin L	
		Cathepsin B	
		Cathepsin H	
Aspartic Proteases	Acidic (pH 2-5)	Cathepsin D	Lysosomes of most cells
		Cathepsin E	

PMN : Polymorphonuclear cells, neutrophils

The proteases of neutrophils are primarily formed during myelopoietic development and stored within a variety of granules. They can be classified according to their activities in antimicrobial defence, protease and phagocytic activities (see Table 2) (Borregaard et al., 1993).

Neutrophil proteases can be released freely from neutrophil granules into the extracellular space: (1) during phagocytosis, (2) by exposure of neutrophils to immune complexes or opsonised substrates (e.g. bacteria) or (3) by pharmacological agents, experimentally. The contents of azurophilic granules may also be freely released from dead or dying cells (Owen and Campbell, 1999). Alternatively, exposure of neutrophils to cytokines and other chemoattractants can result in mobilisation of serine proteases (including NE and CG) to the external surfaces of the plasma membrane (Owen et al., 1995; Owen et al., 1997).

Table 2 Enzymes and other constituents of human neutrophil granules

Granules			
Constituents	Azurophil (Primary)	Specific (secondary)	Small storage (tertiary)
Antimicrobial	Myeloperoxidase Lysozyme Defensins Bactericidal/permeability-increasing protein (BPI)	Lysozyme lactoferrin	
Neutral proteases	Neutrophil elastase Cathepsin G Protease 3	MMP-8 (Collagenase) Complement activator	MMP-9 (Gelatinase B) Plasminogen activator
Acid hydrolases	Cathepsin B Cathepsin D β -D-glucuronidase α -mannosidase Phospholipase A ₂	Phospholipase A ₂	Cathepsin B Cathepsin D β -D-glucuronidase α -mannosidase
Cytoplasmic membrane receptors		CD11b/CD18 CD11c/CD18 fMLP Laminin	

1.3.2 Structure and synthesis of Neutrophil Elastase

Janoff et al. (1968) provided evidence of a neutral protease from extracts of human neutrophil granules that exhibited elastin-degrading activity on vascular basement membranes at physiological pH in vitro and in vivo (Janoff and Zeligs, 1968). They recognised the enzyme as an elastase (Janoff and Scherer, 1968), present in azurophilic (primary) granules of human neutrophils (Dewald et al., 1975). The interest in Neutrophil Elastase (NE) at this time stemmed from its putative role in connective tissue damage (Janoff, 1972; Reilly and Travis, 1980) and its link to the pathogenesis of pulmonary emphysema (Janoff, 1985).

NE (E.C. 3.4.21.37) is a 29.5 kDa cationic glycoprotein (Farley, 1992). Of the serine proteases, NE is regarded as quantitatively the most prominent and potent of all the neutral proteases (Senior and Campbell, 1983). The elastase content of

human neutrophils is quoted as being greater than 10^7 molecules/cell (Plow, 1982). Thus, neutrophils contained in a single millilitre of blood can release over a microgram of NE.

NE exists as four isoenzymes (Baugh and Travis, 1976) with sequence analysis showing that it is a single chain polypeptide of 218 amino acids containing the catalytic triad His-57, Asp-102 and Ser-195 common to all serine proteases (Sinha et al., 1987). The isoenzyme character of NE detected is due to minor differences in carbohydrate content. DNA sequence analysis has been performed to map the NE gene (Farley et al., 1989). Fouret et al. (1989) using in situ hybridisation studies has demonstrated that NE gene expression occurs at the promyelocytic and myelocytic stages of cell differentiation in the bone marrow. NE mRNA levels declined to undetectable levels by the stage of the neutrophil metamyelocyte and thereafter. This provides evidence that gene expression for NE is tightly controlled, with synthesis of NE occurring only in the bone marrow (Fouret et al., 1989). Unlike the MMP's, there is no evidence for pro-forms (zymogens) of NE (Sinha et al., 1987) and it remains in an active form at a concentration of 2-4 μ g/neutrophil (10-12 mM) within the granules. After cellular activation, about 10-12% of the cellular content of NE rapidly translocate from the azurophilic granules to the external surface of the cell membrane (Owen et al., 1997). Using ELISA assays, human NE has also been discovered in the peroxidase-positive granules of a sub-population of monocytes (Campbell et al., 1989; Owen et al., 1994), which can be released in response to injury in much the same way as neutrophil-derived NE. The content of NE identified from monocytes compared to identically prepared neutrophil extracts was 6.2% (Campbell et al., 1989). Macrophages are known to internalise extracellular NE, which casts doubt on the true source.

1.3.3 Physiological roles of Neutrophil Elastase

NE is implicated in the hydrolysis and degradation of a number of extracellular matrix proteins including elastin (Werb et al., 1982), collagen type I (Kafienah et al., 1998), type II (Starkey et al., 1977), type III (Gadek et al., 1980); type IV collagen (Pipoly and Crouch, 1987) and laminin (Heck et al., 1990) in basement membranes; fibronectin (McDonald and Kelley, 1980), and proteoglycans (Malemud and Janoff, 1975). With such broad substrate specificities against

extracellular matrix components, NE may play a significant role in normal wound healing and remodelling (Senior and Campbell, 1983). Despite this, the exact physiological role(s) of NE in normal wound healing is not fully understood (Bieth, 1986). Further biological activities of NE include the inactivation and activation of cytokines and growth factors, cleavage of plasma proteins, coagulation factors and complement components, and lymphocyte and platelet activation (Bieth, 1986; Owen and Campbell, 1999). An overview of these physiological roles is discussed below.

1.3.3.1 Coagulation and fibrinolysis

Allen and Tracy (1995) have demonstrated that NE bound to the cell surface of monocytes catalyses the activation of cofactor Va which itself binds to the monocytes surface and facilitates factor Xa-catalysed activation of prothrombin (Allen and Tracy, 1995). NE has been shown to cleave and inactivate inhibitors of coagulant proteases such as antithrombin III and α_2 -antiplasmin, which may facilitate local coagulation (Sie et al., 1987). NE can also potentiate platelet aggregation (Selak, 1992). Plow (1982) has suggested that NE may be a fibrinolytic enzyme, which can be released from neutrophils in association with coagulation (Plow, 1982).

1.3.3.2 Neutrophil adhesion, chemotaxis and migration

To undertake phagocytosis of cells and necrotic debris, neutrophils must exit the circulation and enter inflamed tissues. There is evidence that NE might play a role in the adhesion and migration of neutrophils from the circulation into sites of tissue injury during inflammation by modulating integrin-mediated cell adhesion.

Zimmerman and Granger (1990) and Woodman et al. (1983) performed in vivo videomicroscopy animal experiments to study the role of NE and its specific inhibitors, Eglin C and L658,758 on neutrophil-endothelial cell interactions and the rate of neutrophil migration in inflamed tissues. Using direct immunofluorescence studies in vitro, Woodman et al. identified that NE induced the surface expression of CD11/CD18 integrin. These investigators proposed that NE might be involved in CD18-dependant chemotaxis and neutrophil accumulation in inflamed tissues.

In further support of a role for NE in mediating neutrophil-endothelial cell interactions, Remold-O'Donnell (1995) used flow cytometry to demonstrate that low levels of NE could specifically cleave CD43, an anti-adhesive surface glycoprotein present on neutrophils that acts to impede neutrophil adhesion to endothelial cells. Related azurophilic proteases (CG and PR3) failed to cleave CD43 (Remold-O'Donnell and Parent, 1995).

In vitro, Cai et al. (1996) showed that NE bound specifically to CD11b/CD18 on neutrophils. They also demonstrated that the active (serine triad) site of NE was involved in the binding to the integrin by measuring the binding of NE to CD11b/CD18 in the presence of a number of serine protease inhibitors, including α_1 -PI and the monoclonal antibody (MoAb), NP57. They provided evidence to show that NE might modulate neutrophil adhesion and migration since blocking NE with the MoAb NP57, prevented detachment, and reduced (by 40%) the chemotactic movement, of neutrophils from a fibrinogen-coated surface. This appears consistent with Woodman's and Zimmerman's interpretation that NE might regulate chemotaxis. Cai et al. (1996) were unable to establish whether NE retained its proteolytic activity when using its active binding site to ligate with CD11b/CD18 (Cai and Wright, 1996).

Cepinskas et al. (1997) assessed the effects of a MoAb against NE and the elastase inhibitor, L658,758 on PAF- and fMLP-induced neutrophil transendothelial migration through HUVEC monolayers in vitro. They reported that both the MoAb and the inhibitor to NE completely inhibited neutrophil migration at low doses of PAF and formyl tripeptide (fMLP, a bacterial peptide analogue). They claimed these in vitro experiments were in agreement with the above in vivo work although similar in vitro work is in contrast with this view (Huber and Weiss, 1989). Nozawa et al. (2000) measured elastase activity using flow cytometry to demonstrate that the adhesion reaction between neutrophils and TNF α -activated HUVEC was dose dependently inhibited by ZD8321, a potent NE inhibitor (Nozawa et al., 2000).

NE has been shown, in vitro, to degrade several components of the vascular basement membranes including collagen (Pipoly and Crouch, 1987), fibronectin (McDonald and Kelley, 1980) and laminin (Heck et al., 1990). Taken together with the above in vitro and in vivo studies, there is strong evidence to support the role

for NE during diapedesis, in which neutrophils could effectively 'digest' their way through the ECM following adhesion to the endothelial wall in inflammation.

1.3.3.3 Antimicrobial properties

Belaouaj (1998) investigated the role of NE in host defence against bacteria in a strain of mice made deficient in NE (NE⁻). They showed that NE⁻ mice were more susceptible to sepsis and death following intraperitoneal infection with the gram-negative bacteria (*Klebsiella pneumoniae* and *Escherichia Coli*) than their normal littermate controls. They claimed that neutrophils migrated normally to sites of infection in the absence of NE, but suggested that NE may be required for intracellular killing by phagocytosis (Belaouaj et al., 1998). Neutrophils and monocytes actively ingest cellular and matrix debris and foreign material into phagosomes, with both acid and serine proteases (including NE) degrading this material following fusion of the azurophilic granules with the phagosome (Owen and Campbell, 1999).

1.3.3.4 Modulation of inflammation

NE can modulate the recruitment of monocytes and leukocytes by inducing the release or activation of cytokines and growth factors from endothelial cells, epithelial cells and monocytes (Owen and Campbell, 1999), and by cleaving them into biologically active forms (Ariel et al., 1998; Coeshott et al., 1999).

With respect to other proteases, NE has also been shown to activate MMP's (Delclaux et al., 1996; Ferry et al., 1997; Nagase, 1997), and inactivate TIMPs (Okada et al., 1988), resulting in an imbalance between MMP and TIMP (Itoh and Nagase, 1995). Despite evidence that NE has a regulatory 'down-stream' role on other proteases, the significance of this behaviour in a pathophysiological setting remains unclear.

Trevani et al. (1997) have studied the effects of serine proteases on apoptosis. Stimulation of human neutrophil apoptosis was observed and quantified in culture in the presence of different concentrations of chymotrypsin, trypsin, and human NE under fluorescence microscopy and flow cytometry. They confirmed that proteolytic activity might be responsible for stimulating apoptosis, since the serine protease inhibitor aprotinin, abrogated the protease-induced apoptosis but they did

not test the use of a specific NE inhibitor to directly confirm a role for NE. They concluded that neutral proteases might have a physiological role in the normal resolution of inflammation (Trevani et al., 1996).

The evidence that the physiological release of NE in tissues for proteolysis may be a controlled process is supported by work from Owen et al. (1995), who were the first to demonstrate that NE could be expressed on the cell surface of neutrophils (Owen et al., 1995). Using a number of pro-inflammatory mediators, these authors reported dose-dependent upregulation of catalytically active surface-bound NE using both immunofluorescence staining and immunogold localization in vitro, whilst unstimulated cells demonstrated minimal expression (Owen et al., 1995; Owen et al., 1997). Catalytic activity of cell-surface NE was confirmed by exposing these same stimulated neutrophils to soluble fibronectin in vitro. Fragments of fibronectin were determined on SDS-polyacrylamide gel electrophoresis (SDS-page). A specific inhibitor to NE (methoxysuccinyl-ala-ala-pro-val chloromethylketone, CMK) inhibited this behaviour. They also claimed that the catalytic activity of the bound NE persisted for at least 3 hours following activation in vitro (Owen et al., 1997). They also demonstrated that α_1 -PI (the naturally occurring inhibitor to NE, with the highest reported association constant for an inhibitor with a protease) was ineffective as an inhibitor to the cell-surface bound NE. When testing other inhibitors of varying molecular sizes in the same system, they noted an inverse relationship between molecular size of inhibitors and their capacity to inhibit surface-bound NE. They concluded that steric hindrance was likely to be the major mechanism by which cell-surface bound NE evaded inhibition from α_1 -PI (Owen et al., 1995).

This work suggests that long-lived catalytically active cell-surface bound NE might be able to focus its powerful extracellular degrading capacity to facilitate neutrophil emigration through the vasculature as well as the penetration of tissue barriers, such as basement membrane (Delclaux et al., 1996), whilst at the same time evading endogenous inhibitors at a pericellular level (Campbell and Campbell, 1988). This mechanism might explain how localised, physiological degradation of ECM proteins could be achieved with minimal injury or disruption to the surrounding tissues in normal growth and repair as well as modulate the functions of other cells, without inciting an inflammatory response through overt injury.

1.3.4 Pathological roles of Neutrophil Elastase

Since NE is the major protease responsible for extracellular proteolysis mediated by neutrophils and that NE has broad substrate specificity, NE has been strongly implicated in neutrophil-mediated tissue injury (Döring, 1994; Owen and Campbell, 1999). In the literature, there is a considerable amount of circumstantial evidence supporting the view that neutrophil-derived NE is implicated in inflammatory disease states and extracellular matrix degradation. NE and its imbalance with α_1 -PI have been historically associated with the development of pulmonary emphysema, cystic fibrosis, Adult Respiratory Distress Syndrome (ARDS), and Rheumatoid Arthritis, which have been extensively reviewed by Janoff (1985) and Bieth (1986).

1.3.4.1 Neutrophil Elastase as a mediator of endothelial cell injury

NE has been reported to play a crucial role in neutrophil-mediated endothelial cell injury and thus contribute to vascular injury (Nakatani et al., 2001; Smedly et al., 1986; Westlin and Gimbrone, 1993). Nakatani et al. (2001) investigated the effects of a number of synthetic protease inhibitors (against the proteases trypsin, plasmin, kallikrein, thrombin and NE) on neutrophil-mediated human endothelial cell injury in vitro (Nakatani et al., 2001). Endothelial cell lysis was measured by the release ^{51}Cr -labelled HUVEC following neutrophil priming with TNF- α . Of the serine protease inhibitors tested in this model system, only ONO-5046 (a specific synthetic inhibitor of NE) decreased HUVEC cytotoxicity compared to controls. A role for elastase in inducing endothelial cell injury was confirmed by incubating purified NE with stimulated ^{51}Cr -labelled HUVEC. NE increased the % lysis of HUVEC in a dose-dependent fashion, which was further negated by ONO-5046 (Nakatani et al., 2001). These results strongly implicated NE as a mediator of endothelial injury in vitro.

Investigators have attempted to demonstrate a clear correlation between increased neutrophil-derived NE and the clinical course of a disease or injury, suggesting that increasing levels of NE might mediate the intensity of the

inflammatory process. A number of clinical studies have been published to evaluate whether plasma levels of NE might be a good marker for determining disease activity such as psoriasis (Orem et al., 1997), provide a predictor of MOF (Jochum et al., 1994; Nast-Kolb et al., 1997), post-operative marker of wound healing complications (Hofer et al., 1995) or a predictor of wound healing in chronic wounds (Hoffman et al., 1999).

1.3.4.2 Evidence of direct Neutrophil Elastase-mediated tissue damage in skin

In vitro studies have directly demonstrated structural epidermo-dermal damage caused by NE. Briggaman et al. (1984) removed split-thickness skin from human volunteers and subjected them to purified human NE for periods of 2-8 hours at concentrations of between 100 and 700nM, which might be found under physiological and pathological conditions in skin respectively. Using electron microscopy (EM), they noticed a decrease in the density of the lamina densa, within 2 hours compared to controls, which was absent by 6 hours. The lamina densa of blood vessels was also destroyed, which was not observed for other serine proteases. Distinctive epidermal-dermal separation was noted. Immunohistochemical staining of collagen type IV and laminin were absent which they suggested was an indication of profound dissolution of the basal lamina (Briggaman et al., 1984).

A similar study was also performed confirming epidermo-dermal junction damage with widening of intercellular spaces and the disappearance of the basal lamina on EM (Glinski et al., 1991).

Ludolph-Hauser et al. (1999) have demonstrated widening of the keratinocyte intercellular spaces under EM, in vitro, when NE was applied topically at concentrations as low as 3nM. The difference in this study was that the horny layer was tape-stripped to ensure penetration of NE through the lower keratinocytes, and the skin was cultured to remove contamination by serum-derived proteases inhibitors (Ludolph-Hauser et al., 1999).

The exact local elastase concentration in skin during tissue injury is not known, but these studies at least demonstrate the directly damaging effects of NE on skin constituents.

1.3.4.3 Elevated Neutrophil Elastase in tissues of non-healing skin wounds

Herrick et al. (1997) assessed the protease activity in wound tissue derived from chronic venous ulcers compared to control acute wound biopsies. Tissue extracts were obtained by homogenising the tissue samples in buffer solution before centrifuging the supernatant. They determined that the predominant protease responsible for fibronectin degradation (identified by fibronectin zymography) in both acute wound tissue extracts and venous ulcer tissue extracts was a serine protease, since this activity was abolished by AEBSF (a broad range serine protease inhibitor) but not by EDTA (a metalloproteases inhibitor). Immunoblotting studies using a specific anti-neutrophil elastase antibody confirmed that this protease activity seen on fibronectin zymography was NE. They also demonstrated that NE activity was upregulated in chronic wounds and acute wounds of aged subjects compared to acute wounds of younger subjects. Specifically, they showed that the NE activity increased from the surrounding “normal” skin to the ulcer base in the biopsies of chronic wounds in the same subject. This finding correlated with immunohistochemical staining for NE, showing that elastase staining was more prevalent in the ulcer base and edge than in the surrounding skin and present in greater quantities in acute aged subjects compared to younger controls. Since there were no age- or gender-related differences in elastase activity in circulating neutrophils and that the total number of neutrophils measured in the peripheral blood did not significantly vary with age, they suggested that the elevated elastase activity present in wounds was due to a greater number of neutrophils infiltrating the wound site (Herrick et al., 1997).

The actual mechanism by which NE might achieve neutrophil locomotion through the extracellular matrix remains unclear as well as the degree to which the presence (and action) of NE contributes to ‘normal’ physiological or pathological inflammatory roles.

1.3.5 Inhibitors of Neutrophil Elastase

1.3.5.1 Alpha -1 protease inhibitor

Alpha-1 protease inhibitor (α_1 -PI), formally known as α_1 -antitrypsin, is a 51 kDa serine protease inhibitor (SERPIN) consisting of 394 amino acids and three carbohydrate chains that can diffuse readily throughout the interstitial fluids. It has a single active site at Met³⁵⁸ – Ser³⁵⁹ and is capable of binding avidly to serine proteases, most notably with NE (Jeppsson, 1996).

The main production of α_1 -PI resides in the hepatocytes of the liver. Synthesis of α_1 -PI is increased (up to 4-fold) as part of the acute phase response, notably in inflammatory reactions and following trauma (Gabay and Kushner, 1999). Synthesis and storage of α_1 -PI has been confirmed in monocytes, alveolar macrophages and alveolar and intestinal epithelial cells (Jeppsson, 1996).

Surprisingly, α_1 -PI gene expression, synthesis and secretion have also been demonstrated in neutrophils (Du Bois et al., 1991). Furthermore, Mason et al. (1991) carried out ultrastructural immunohistochemical double staining of neutrophils with antibodies directed towards NE and α_1 -PI to reveal colocalisation within the azurophil (primary granules) of the cells (Mason et al., 1991). Pääkkö et al. (1996) used immunofluorescence microscopy with antibodies against α_1 -PI on neutrophils, with CD3 double staining to delineate monocytes. They demonstrated α_1 -PI in the cytoplasm of all neutrophils, with fewer staining in monocytes (Pääkkö et al., 1996). From the work by Du Bois and Pääkkö, neutrophil-derived α_1 -PI may provide an important role in regulating the local release of NE following its activation. The current view is that the amount of α_1 -PI synthesised, stored and released from neutrophils, alone, would not be large enough to overcome the massive tissue destruction by NE in vivo; instead, neutrophil-derived α_1 -PI serves as a local modulator of neutrophil diapedesis or to provide protection from cellular leakage during phagocytosis.

α_1 -PI is the predominant endogenous inhibitor to NE in plasma, capable of diffusing from the circulation into the interstitial space (Travis and Salvesen, 1983). An understanding of the role of α_1 -PI has been gained from observing patients with α_1 -PI deficiency, in which emphysema develops because of inappropriate and uncontrolled NE-mediated elastolytic lung injury (Janoff, 1985). Elimination of

extracellular NE occurs through the formation of a tight stable 1:1 complex with α_1 -PI, which is later removed and catabolised within the circulation (Carrell, 1986).

Oxidative (Clark et al., 1981; Ossanna et al., 1986) and proteolytic inactivation (Michaelis et al., 1990; Zhang et al., 1994) of α_1 -PI can occur. Beith (1986) reported that oxidation of α_1 -PI resulted in a 2000-fold decrease in the inhibition rate for NE (Bieth, 1986), the implication being that an imbalance of protease/anti-protease would favour NE-mediated tissue destruction.

In addition to its important protease inhibitory functions, α_1 -PI has also been reported to influence neutrophil behaviour via both direct and indirect mechanisms. There is evidence that α_1 -PI may be responsible for modulating the migratory capacity of neutrophils to sites of inflammation. Proteolytically modified α_1 -PI (Banda et al., 1988b) and α_1 -PI/NE (Banda et al., 1988a) complexes and can act as potent chemoattractants to neutrophils and may play a role in sustaining inflammatory responses at sites of tissue injury.

1.3.5.2 Skin-derived antileukoproteinase (SKALP/ELAFIN)

SKALP is a skin-derived protease inhibitor (Mr of 10 kDa and 20 kDa) with specificity for NE and PR3 (Schalkwijk et al., 1990). Others have coined the term ELAFIN to describe a similar (if not the same) Elastase specific inhibitor with a Mr of 6kDa (Wiedow et al., 1990). The kinetics of the inhibition of NE by ELAFIN suggests that it is a potent but fully reversible skin-derived inhibitor of NE (Ying and Simon, 1993). Whilst it is absent in normal human skin, SKALP/ELAFIN is strongly induced in inflammatory skin diseases (van Bergen et al., 1996) .

1.3.5.3 Secretory leukocyte proteinases inhibitor (SLPI)

SLPI is another human NE inhibitor of between 10 and 14 kDa, which has been identified in peripheral blood neutrophils. Sallenave et al. (1997) assessed the anti-NE activity of SLPI from neutrophil cytoplasm to determine its activity in vitro. They suggested that the binding characteristics of NE/SLPI at a molar equivalence of 80:67.8 (= 1.12) strongly supported the view that SLPI was the main active inhibitor of NE in the neutrophil cytoplasm. SLPI can also be secreted from the cells upon stimulation. Although α_1 -PI is also present in neutrophils, Sallenave et al. (1997) claimed that it represented as little as 0.8% of the molar amounts of

SLPI, but they did not provide any data to substantiate this. They reported that ELAFIN was also present in neutrophil cytoplasm but at 0.05% of SLPI levels and suggested that both α_1 -PI and ELAFIN were unlikely to be important contributors to the NE-inhibitory capacity of the neutrophil (Sallenave et al., 1997).

Little is known about the functions of both SKALP/ELAFIN and SLPI, but they appear to represent a set of highly specific regulators of NE at the level of the neutrophil cytoplasm, whose function might be to protect the cell from NE leakage from the primary granules. In addition to this, skin-derived SKALP/ELAFIN has also been demonstrated within keratinocytes during wound healing but the functional significance of this has yet to be fully elucidated.

1.3.6 The balance of Neutrophil Elastase and inhibitors

Owen and Campbell (1999) have reviewed the methods that might allow proteases to circumvent the effects of high-affinity protease inhibitors that are present in the extracellular space (Owen and Campbell, 1999).

1.3.6.1 Inactivation of protease inhibitors

Inhibitors can be inactivated by oxidative or proteolytic mechanisms (Reddy et al., 1994; Weiss and Regiani, 1984). For example, MMPs can inactivate α_1 -PI (Michaelis et al., 1990) and NE can inactivate TIMPs (Itoh and Nagase, 1995; Okada et al., 1988). Therefore, activated neutrophils might be able to create microenvironments in which extracellular proteolysis is facilitated via inactivation of inhibitors.

1.3.6.2 Adherence and compartmentalization

Neutrophil proteolysis can occur when cells are in direct contact with the substrate, despite the presence of protease inhibitors in the immediate pericellular environment (Campbell and Campbell, 1988).

1.3.6.3 Tight binding of protease to substrate

In vitro studies have demonstrated that NE binds stably to elastin, and that α_1 -PI and Secretory leucoprotease inhibitor (SLPI) have reduced effectiveness against the catalytic site of NE when bound (Morrison et al., 1990).

1.3.6.4 Membrane binding of proteases

In contrast to NE and CG that are freely released from leucocytes, it has been shown that membrane-bound NE and CG demonstrate a resistance to inhibition by naturally occurring inhibitors (such as α_1 -PI) (Owen et al., 1995). The mechanism by which this is achieved remains unclear. This work suggests that binding of proteases to the cell surface focuses and preserves their catalytic activity to the immediate pericellular environment, even in the presence of inhibitors.

1.3.6.5 Overwhelming of inhibitors

NE may overwhelm inhibitors by releasing massive quantities of enzymes from large numbers of persistently activated inflammatory cells (as in the case of rheumatoid arthritis, cystic fibrosis or abscesses, for example). Alternatively, high concentrations of NE may be released from individual cells. Liou and Campbell (1996) reported that quantum bursts of NE-mediated proteolytic activity occurs when neutrophils release their azurophilic granules following their adherence to, or migration on, opsonised matrix proteins. The brief catalytic activity exceeds the concentrations of local extracellular inhibitors until diffusion of the granule contents allows the ratio of NE: α_1 -PI concentration to drop to less than a 1:1 ratio (Liou and Campbell, 1996).

1.3.7 Neutrophil Elastase and extracellular matrix degradation

The extracellular matrix (ECM) is no longer viewed as a supportive structure and physical barrier, but instead an active zone that functions to instruct cellular differentiation by initiating signal transduction pathways and controlling the

activities of cytokines and growth factors (Streuli, 1999). Therefore remodelling of the ECM during wound healing can have profound effects on its function and the behaviour of the cells that reside within it.

Controlled degradation of the ECM is required for the removal of damaged components of tissue injury, dissolution of the basement membrane and to allow cell migration and angiogenesis (Clark, 1996). Neutrophil extravasation through the ECM during inflammation appears to be dependent on neutrophil-derived proteases (Murphy and Gavrilovic, 1999).

Excessive destruction of ECM molecules by neutrophil-derived proteases has been implicated as a contributing factor in a variety of acute and chronic inflammatory states (Yager and Nwomeh, 1999), and the failure of wounds to heal (Falanga et al., 1995). For example fibronectin, which is an important ECM protein required for wound repair (Clark et al., 1982), appears very susceptible to NE degradation (McDonald and Kelley, 1980; Wysocki and Grinnell, 1990), the fragments of which can even induce the release of more elastase from activated neutrophils (Wachtfogel et al., 1988).

1.4 Wound Fluid Analysis in Wound Healing Research

Wound fluid provides a useful means of understanding wound pathophysiology. The wound exudate is the medium in which wound repair takes place, the contents of which is believed to reflect the status of the wound (Falanga, 1992; Hunt, 1991). An assumption has been made that the biochemical composition of wound fluid represents the extracellular environment of the wound, and is supported by studies that have profiled electrolytes and proteins from paired wound fluid and serum taken from healing and non-healing chronic wounds and burns (Trenegove et al., 1996; Young and Grinnell, 1994). The wound fluid bathing the cells and extracellular matrix proteins within the wound environment provides a valuable source of quantitative information about the in vivo activities of different wounds at a local level. Analysis of wound fluids from acute and chronic wounds may identify different cell types, matrix proteins and their fragments, and biological factors (including growth factors, cytokines, proteases and their inhibitors), which either impair or facilitate wound healing (Tarnuzzer and Schultz, 1996; Yager et al., 1997). Many wound fluid studies have been performed which aim to identify differences in wound constituents between acute and chronic wounds (Falanga, 1992; Trenegove et al., 1999), between different acute wound types (Baker and Leaper, 2000) and chronic wound types (Trenegove et al., 1999).

1.4.1 The significance of wound fluid analysis

The wound fluid milieu can change rapidly over time, reflecting the dynamic changes between cell to cell, and cell to matrix interactions. Tarnuzzer and Shultz (1996) suggested that an impairment or imbalance in wound environments might promote and maintain the chronicity of wounds (Tarnuzzer and Schultz, 1996). Different investigations into the protease activities within wound fluid provide evidence that different proteases are participating at different stages of wound healing. The integrated, functional activities of proteins expressed (or not) within wound fluid might yield clues as to the factors responsible for healing or non-healing wounds.

Whilst biopsies may be homogenised for analysis of the tissue or cellular extracts, the technique of fluid extraction from biopsy material in wound healing research is limited because: (1) repeated biopsy sampling within the same patient precludes frequent sampling; (2) biopsy sampling itself might change the nature of the wound environment; (3) homogenised extracts of the cells and tissues may not provide an accurate reflection of the changing extracellular fluid environment; (4) careful standardization of the methods is required. Young and Grinnell (1994) have suggested that the analysis of the human wound environment using wound fluid provides information similar to that obtained when tissue is excised and analysed (Young and Grinnell, 1994).

1.4.2 Methods and models used in wound fluid analysis

Models can be classified into artificial material models (in which something is placed onto or into a wound) or tissue models (the wound can be created or manipulated for wound fluid collection; for example, blister wounds) (Gottrup et al., 2000). A review of some of the more common methods by which researchers have collected or analysed wound fluid in vivo is described below.

1.4.2.1 Chamber models

A wire mesh chamber model placed subcutaneously into wounds becomes filled with wound fluid and connective tissue matrix components (Hunt, 1967). Reliable samples can be obtained from the wound chambers after seven days, and continuous sampling can be carried out for weeks and or months. The advantage of this model includes the frequent sampling of large quantities of tissue fluid and granulation tissue. Ehrlich et al. (1983) used a stainless steel mesh chamber to collect repeated fluid samples from experimentally induced burn and freeze injuries to the skin in rats (Ehrlich et al., 1983). Hurtenbach et al. (1995) have used a perforated 30 x 10 mm polytetrafluoroethylene (Teflon) tissue chamber implanted subcutaneously in mice to collect wound fluid following infection-induced inflammation (Hurtenbach et al., 1995). The disadvantages of these implanted

chambers are their size and the need for surgical placement and the irritant nature of the material used.

Open wound fluid collection chambers is a common way of collecting wound fluid (Breuing et al., 1992; Marikovsky et al., 1993). Breuing et al. (1992) developed a surface wound chamber with a flexible transparent vinyl top bonded to an adhesive vinyl base (Breuing et al., 1992). Vogt et al. (1998) adapted Breuing's design to collect donor site wound fluid from humans (Vogt et al., 1998). The device was mounted on silicone sheets with a 15 x 15 mm opening directly onto the wound surface. After split-skin excision, the apparatus was placed onto the wound, and the chambers were filled with Normal Saline and sealed. After 24 hours, the total fluid was aspirated and replaced with a further Saline.

The problem with collecting chambers concerns the length of time the wound fluids or diluent fluid is left in situ. This might lead to problems of interpretation given that many of the constituents of wound fluid may breakdown or digest other endogenous factors (Egger et al., 1997).

1.4.2.2 Subcutaneous tubes

A subcutaneous implanted porous expanded polytetrafluoroethylene (ePTFE) tube can be implanted into the skin or wound using a trochar (Goodson and Hunt, 1982). A standard tube of 90 to 120 μm porosity, with a 1.2mm internal diameter and 0.6mm wall thickness can be bought commercially. The tube allows for diffusion of gas and fluid through the device. It is commonly used to promote granulation tissue formation and for the study of collagen and inflammatory cells. The disadvantage is that it is less good for the study of early wound healing, and does not represent a good way of collecting fluid. Haukipauro et al. (1987) described the use of a silicone rubber device to collect up to 16 samples over 1-5 days using this technique (Haukipauro et al., 1987). Despite this, there is a delay between fluid production and collection.

1.4.2.3 Sponges

The polyvinyl alcohol sponge model incorporates a sponge placed inside a perforated silicone tube (Diegelmann, 1986). Implanted using a needle and trochar technique, the model is minimally invasive and acceptable to patients. The

Cellstick[®] is a similar model which consists of a silicone material containing a viscose cellulose sponge implant, allowing fluid to exit the wound continuously, behaving much like a minor wound drain (Viljanto, 1976). The main advantage of these models is the suitability in studying wound fluid components in the early phase of wound healing. Inflammatory cells can also be collected. The disadvantages are the complicated interpretations of the results and the experience required for its use.

1.4.2.4 Dressings

Polyurethane adhesive dressings are common ways of trapping donor site wound fluid or chronic wound fluid (Grayson et al., 1993; Ono et al., 1995a). The problem with this technique is that the wound surface area from under the dressing is not standardised and the collection of wound fluid from under these dressings has to be taken after a period of some hours before a sizeable volume can be obtained. Other issues concern the frequency with which fluid collection is obtained. Some investigators have used methods to manually express wound fluid from dressings (Rao et al., 1995) or soaked them in buffered detergents (Hoffman et al., 1998) in order to collect their contents. Each technique may create discernible differences when measuring the activities of proteases from wound fluids.

1.4.2.5 Drainage bottles

Collection of acute wound fluids from operative sites (such as breast and abdomen) using drainage bottles under gravity or suction is widely reported in the literature (Nwomeh et al., 1999; Tarlton et al., 1997). Despite the ease of collection, significant disadvantages include the time taken before the sample is collected and stored, mixing over a number of hours, failure to collect over ice and the possible contamination by blood.

1.4.2.6 Cutaneous microdialysis

The microdialysis technique makes it possible to investigate the dynamic behaviour of compounds made up of smaller molecules in the environment of the

wound (Church et al., 1997). A typical microdialysis probe has an outer diameter of about 200µm, a wall thickness of 8µm and a molecular weight cut-off of 2 kDa. Whilst not a technique for collecting wound fluid per se, these probes can perform precise quantitative measurements of the concentration of substances (usually drugs or chemical mediators) in the immediate vicinity of the probe. The disadvantages are that large proteins cannot be measured given their size, the sample volumes are small and the cost of equipment and the expertise required is considerable.

1.4.2.7 Miscellaneous methods

Paloahti et al. (1993) collected chronic wound fluid exudates using blunt glass microcapillaries, but only small amounts of fluid could be retrieved for analysis using this method (Palolahti et al., 1993). Cooper et al. (1994) have used inert porous dextranomer beads placed in patient's pressure ulcers over 24 hours to recover endogenous growth factors from chronic wounds (Cooper et al., 1994). Ono et al. (1995) have directly aspirated fluid from blisters of human burns to gain insights into the make up of burn wound fluid constituents (Ono et al., 1995b). The problem with this is that usually only one sample can be collected from the wound site, which makes comparisons difficult within and between patients over time.

1.4.3 Proteases and their inhibitors in wound fluid

1.4.3.1 Protease and anti-protease profiles in acute wound fluid

Moses et al. (1996) utilised SDS-PAGE, radiometric assays and Western blot analyses to determine the temporal pattern of appearance and activity of MMPs and their inhibitors during the healing process in a partial thickness wound model in four pigs over a 10 day period (Moses et al., 1996). They placed 1.2mls Normal Saline containing penicillin and streptomycin into an open wound chamber device described by Breuing et al. (1992). The fluid was retrieved every 24 hours and further fluid replaced. They observed no MMP activity in the first hour samples. The most prominent proteolytic activity, which was observed on each day,

migrated at 94 kDa on SDS-PAGE, consistent with the pro-form of MMP-9. They never observed active MMP-9 in any of their wound fluid samples. They suggested that the pattern of appearance is consistent with the time course of appearance of the inflammatory cells, neutrophils and macrophages that are known to store and release MMP-9 (Owen and Campbell, 1999).

Tarlton et al. (1997) collected acute wound fluid (AWF) from the drains of 9 patients undergoing surgery for mastectomy (for up to 96 hours after operation), and 9 patients after colectomy (from 24 to 120 hours after operation). Collections were obtained via sterile drainage systems over six time points. Using gelatin zymography, these authors reported the predominance of MMP-9 and MMP-2 pro-forms and a MMP-9 complex in their AWF, with no differences in levels between the two wound groups. The proMMP-9 levels peaked at 24 hours, which then fell significantly over the 96 and 120-hour time points. Interestingly, they found that proMMP-9 levels did not drop in infected mastectomy fluids, reaching 2.2 times greater than non-infected acute mastectomy fluid. Casein zymography was used to identify NE, with corroboration of identification using both PMSF (a serine protease inhibitor) and elastatinal (a specific NE inhibitor). No activated MMP-2, MMP-9 or NE was detected in normal acute drain wound fluids using these tests (Tarlton et al., 1997; Tarlton et al., 1999).

Further evidence suggesting that latent MMP-2 and MMP-9 are elevated in acute human wound fluid compared to plasma has been derived from a number of studies of post-mastectomy and post-abdominoplasty or post-hernia drainage fluids (Agren et al., 1998; Bullen et al., 1995; Wysocki et al., 1993; Yager et al., 1996). The major problem with studies that involve surgical drainage fluid is that the wound fluid comprises a mixture of blood, serum, lymph and inflammatory exudate from muscle and subcutaneous tissue, which is unlikely to resemble dermal wound interstitial fluid and so results of these cases have to be interpreted with a degree of caution with respect to human dermal wounds. Despite this, all these studies showed that the amount of MMP-9 (predominantly derived from neutrophils) was relatively higher than MMP-2 (secreted mainly from fibroblasts), which is consistent with the established pattern of large numbers of neutrophils in both wounds and wound fluid obtained during the early inflammatory phase following wounding.

Nwomeh et al. (1998) examined exudates from under occlusive film dressings covering full-thickness skin biopsy sites in 20 healthy human volunteers. Exudate fluid was collected on days 1,2,3,4, 7 and 8. Using ELISA kits for MMP-1 and MMP-8 (which is almost exclusively derived from neutrophils) they demonstrated quantitatively more MMP-8 than MMP-1 (up to 200-fold) in these wounds, with peak levels of MMP-8 in dermal exudates occurring on day 4, and peak MMP-1 levels on day 7. They argued that their data, showing significantly increased levels of MMP-8, reflect the numbers of neutrophils in the wound at this time (Nwomeh et al., 1998).

Nwomeh et al. (1999) compared MMP-1 and MMP-8 levels taken from the same cutaneous wound fluid exudates (n=20) with acute surgical wound fluid taken from drains of patients (n=4) who had undergone either mastectomy or myocutaneous flap reconstruction procedures. At each corresponding time point (days 1,2,3,4,7 or 8) and using the same ELISA assay, they found that the levels of both collagenase were significantly lower in surgical drain fluids than in fluids collected from healing dermal wounds. Again, regardless of wound type, MMP-8 was at least 100-fold higher than MMP-1 at all time points (Nwomeh et al., 1999). This study clearly highlights the limitations when comparing the same wound fluid constituents from different acute wound fluids (such as dermal exudate and drainage fluid from surgical wounds).

Bullen et al. (1995) analysed AWF taken from 5 drains of patients following mastectomy every 12 hours for up to 10 days post surgery. Gelatin zymography and immunoprecipitation identified relative changes in the temporal profiles of MMP-2 and MMP-9. MMP-9 was consistently greater throughout the 10 days but gradually dropped over this time. TIMP-1 levels were also measured and found to increase 10-fold over the first two days among the five patients studied. Hence, the ratio of MMP-9 to TIMP-1 rapidly decreased in acute wound fluid, which they suggested might represent a shift in the wound environment from one favouring tissue turnover to one favouring matrix deposition (Bullen et al., 1995).

Some studies have measured the activity of the serine proteases in AWF and compared them to chronic wound fluids (discussed in section 1.4.3.2 below). However, three studies are worthy of mention.

Rao et al. (1995) collected AWF (n=4) from under occlusive dressings covering donor site wounds and from breast suction drains, and quantified the NE activity.

NE activity was comparable to normal human serum samples, but remained significantly lower than chronic wound fluid samples (see below) (Rao et al., 1995).

Yager et al. (1997) measured the NE activity from AWF (n=10) taken from the drains of 5 patients following abdominoplasty. They also found that the NE activity levels were comparable to plasma levels but remained significantly lower than the NE activity of chronic wounds (see below) (Yager et al., 1997).

Weckroth et al. (1996) collected AWF from six donor site wounds on the operation day, and postoperative days 3 and 5. They provided evidence suggesting that both NE and CG activity was greater than serum and chronic wound fluid. However, they clearly demonstrated that AWF showed remarkable variation between their six patients, with rapidly changing activities of both serine proteases between different postoperative days (Weckroth et al., 1996).

1.4.3.2 Protease and anti-protease profiles in chronic wound fluid

The protease profile of chronic wound fluid (CWF) has been studied extensively over the last 10 years in an attempt to understand the pathophysiology of chronic wounds. An interest in studying CWF was fuelled by the discovery that the mitogenic activities of acute and chronic wound fluids differed (Bucalo et al., 1993; Katz et al., 1991) and that wound fluid samples from chronic wounds were found to contain degraded adhesion proteins (Wysocki and Grinnell, 1990), indicating that the wound environment might contain active proteases. Attention has focused on the discovery of increasing amounts of neutrophil-derived, matrix-degrading enzymes present in CWF that are released into the wound environment following activation of neutrophils during wounding. Recent studies suggest that the main groups of protease include the metalloproteases, MMP-1 and MMP-8 (Nwomeh et al., 1999), MMP-2 and MMP-9 (Bullen et al., 1995; Wysocki et al., 1993; Yager et al., 1996), and the serine proteases, Plasmin (Palolahti et al., 1993), and NE (Grinnell and Zhu, 1996; Hoffman et al., 1998; Rao et al., 1995). These papers all reported greater protease levels in chronic than acute wound fluids, although these findings are not universal (Trenegove et al., 1999; Weckroth et al., 1996).

Concomitant studies have also shown that linked to their respective proteases, the presence of both TIMPs (Bullen et al., 1995; Nwomeh et al., 1999; Trenegove et al.,

1999) and α_1 -PI (Grinnell and Zhu, 1996; Rao et al., 1995; Yager et al., 1997) in CWF were reduced or degraded when compared to AWF.

With particular reference to NE, Rao et al. (1995) analysed the activity of elastase retrieved from 10 chronic ulcers and compared them to AWF (from donor sites or drains following mastectomy). CWF samples contained 10- to 40-fold more elastase activity than AWF or serum. Immunoblot analysis of these CWF samples revealed that fibronectin and α_1 -PI were degraded in 9 out of 10 samples, but were intact in all the AWF samples. Furthermore, they suggested that MMPs were not responsible for fibronectin degradation in their chronic wounds, since incubation of the fluids with fibronectin in the presence of EDTA (a potent inhibitor of MMPs) did not prevent the appearance of specific fibronectin fragments. Following incubation of fibronectin in the presence of CWF samples accompanied by a number of serine-, cysteine-, and acid-protease inhibitors, they discovered that fibronectin degradation was completely inhibited by PMSF (a specific inhibitor of serine proteases) only. Given the elevated levels of elastase present in their CWF samples, they suggested that it was this serine protease that was responsible for fibronectin degradation. They supported this with further evidence demonstrating that the degradation of fibronectin by their CWF proteases was indeed inhibited by α_1 -PI and not by other SERPINS (Rao et al., 1995).

Grinnell and Zhu (1996) examined fibronectin degradation and NE levels in wound fluid sampled longitudinally from 3 patients with venous ulcers. CWF was obtained at weekly intervals over a period of four weeks, obtained after a 4-hour period under occlusive dressings. AWF comparisons were made with mastectomy fluid. These authors noted that fibronectin degradation patterns were unique to different patients, and that this was occurring locally since fibronectin was intact in paired plasma samples taken from the same patient. They demonstrated that elastase activity was elevated in all the wound fluid samples that contained degraded fibronectin, with little detectable elastase activity found in those samples that contained mostly intact fibronectin. Moreover, they demonstrated a close correlation between the presence of elastase activity and the appearance of fibronectin fragments in CWF. Despite high levels of MMP-9 profiles present in these same CWF samples which also appeared to correlate with fibronectin degradation, specific elastase inhibitors (ICI 200,355 or MDL 27,367) as well as the general serine protease inhibitor AEBSF, blocked the ability of their CWF to

degrade fibronectin. On the other hand, the metalloproteinase inhibitor, EDTA, had no effect. Grinnell and Zhu also performed experiments to determine if the inhibitors α_2 -macroglobulin and α_1 -PI were present in their ulcer wound fluid. They showed both of these inhibitors had undergone significant cleavage in those same samples that contained fibronectin fragments. In vitro, increasing concentrations of human NE was added to acute mastectomy fluid and fibronectin and gelatinase profiles of this AWF determined. In the absence of NE, fibronectin was intact and elevated levels of MMP-9 and its complexes were evident. With increasing concentrations of NE, fibronectin was completely degraded and interestingly MMP-9 appeared to be cleaved from its proenzyme form. When NE was incubated with mastectomy fluid in the presence or absence of EDTA (to block MMP activity), the pattern of fibronectin degradation was similar. These results also implicate wound fluid Elastase as the dominant protease in fibronectin degradation in CWF, which appeared to regulate MMP-9 activity too. The drawback with this study is that only 3 patient CWF samples were analysed (Grinnell and Zhu, 1996).

Although definitive evidence directly implicating NE as the dominant protease present in CWF falls short in these studies by Rao et al. (1995) and Grinnell et al. (1996), the circumstantial evidence is compelling. They also provide evidence that an imbalance of NE and its potent endogenous inhibitor, α_1 -PI, is present in CWF which may contribute to the enhanced NE activity noted, since Rao et al. (1995), Grinnell et al. (1996) and Yager et al. (1997) all confirmed that α_1 -PI was degraded in these fluids compared to AWF controls.

1.4.3.3 Protease and anti-protease profiles in burn wound fluid

In contrast to CWF, studies that have examined proteolytic activity in human burn wound fluid are few. There is evidence that the NE activity in blood samples following burn injury is increased sharply (Miskulin et al., 1978; Neely et al., 1992), that this is proportional to the extent of the burn (Barisoni et al., 1991; Neely et al., 1988; Ozkan et al., 1988), and that an imbalance with α_1 -PI has been noted (Faymonville et al., 1987). There is also evidence to show that protease activity in burn tissue is increased compared to unburned skin (Neely et al., 1997; Stricklin and Nanney, 1994).

The paucity of data available on burn wound fluid may be due to the practical difficulties of collecting the fluid from burn wound surfaces. MMP activity has been noted in burn wound fluid (Grinnell et al., 1993; Grinnell et al., 1998), especially that of MMP-9, consistent with neutrophils as the initial source of this enzyme (Young and Grinnell, 1994). Serine proteases have also been identified (Grinnell and Zhu, 1994; He et al., 1998).

Grinnell and Zhu (1994) provided the most recent literature on NE in burn wound fluid. They analysed wound fluids from 11 patients who had either superficial or deep partial thickness burns or full thickness burns using zymography, immunoblotting studies and a human NE activity assay. One burn wound fluid sample was taken from each patient from between days 1 to 8 by trapping burn fluid under a sterile glove over a 4-hour period. Compared to paired plasma samples, which contained intact adhesion proteins, all the burn fluids had evidence of fibronectin or vitronectin degradation in variable amounts, with particularly large amounts of degradation in three cases. Using zymography, all burn wound fluids contained proteases (MMP-9 and MMP-2), the highest levels corresponding to the three burn wound fluids with the greatest fibronectin degradation. However, when they performed the NE activity assay on the same three burn wound fluids, they also showed high elastase activity.

Using protease inhibitors to block fibronectin degradation, they demonstrated that the protease activity was blocked most effectively using AEBSF (the broad spectrum serine protease inhibitor) with degradation being unaffected by o-phenanthroline or EDTA (MMP inhibitors), or by the serine inhibitor aprotinin or leupeptin (trypsin class of serine protease blockers). From this work, they suggested that elastase or the chymotrypsin class of serine protease (like CG) might be primarily responsible for fibronectin degradation.

In vitro, samples of fibronectin were then added to the burn wound fluids shown previously to have excessive fibronectin degradation, along side purified human NE as a control, and demonstrated similar fibronectin degradation profiles, which they suggested was consistent with NE as the primary protease causing fibronectin degradation in human burn wound fluid. This finding is also consistent with reports that have described the ability of NE to degrade fibronectin in vitro (McDonald and Kelley, 1980).

In order to rule out CG as a possible contender, they compared fibronectin degradation by elastase and CG and wound fluid in the presence of specific NE inhibitors (ICI 200,355 and MDL 27,367) or chymostatin (inhibitor of chymotrypsin-related serine protease, CG). The results showed that the activity of the protease responsible for burn wound fluid fibronectin degradation was blocked by NE inhibitors but not by the CG inhibitor. Indeed, fibronectin degradation profiles with synthetic NE in the presence of the same inhibitors matched those of burn wound fluid. In other experiments, a series of fluid samples from one burn were incubated with polyclonal antibodies against human NE and tested for fibronectin degradation. They reported that addition of the anti-NE antibody completely neutralised fibronectin degradation by NE or burn wound fluid but had no effect on fibronectin degradation by CG.

Finally, after attaching fibroblasts on to fibronectin-coated cell culture dishes, they applied burn wound fluid which caused the fibroblasts to round up and stop spreading, an activity that could be neutralised by the NE inhibitors (ICI 200,355 and MDL 27,367).

From this detailed work, these authors concluded that NE was the most likely protease responsible for fibronectin degradation in burn wound fluid (despite the presence of active MMPs and other serine proteases) and that its presence in burn wound fluid was able to interfere directly with fibroblast adhesion to fibronectin. The high elastase activity measured in the burn wound fluid samples could not be detected at all in the corresponding plasma, which they interpreted as suggestive of local production and release of active NE from migrated neutrophils (Grinnell and Zhu, 1994).

Unfortunately, much of this work was focused on just three out of the 11 (one-off) burn wound fluid samples in light of their marked protease activities and fibronectin degrading profiles. They did not explain why the pattern of protease activity on fibronectin degradation was not universal to all fluid samples, but they did concede that differences might be because of (or a combination of) different classes of enzymes.

1.4.4 Considerations and cautions in wound fluid analysis

The biochemical characterization of wound fluid can be difficult to interpret. The exact composition depends on the mode of injury, the wound itself, and the collection technique (Falanga, 1992). It is essential to standardise the methods and storage of fluid collection, for this will have a direct impact on the composition of the fluid. Although peripheral blood samples were used as an example, Egger et al. (1997) demonstrated the variability of NE measured in samples of 5 patients, which were subjected to differences in storage time, temperature and agitation. They reported significant increases in NE measurements with time (5 minutes to 24 hours) and concluded that in order to obtain reliable results from PMN functional tests, processing of samples should occur within 20 minutes after blood withdrawal (Egger et al., 1997).

In some of the studies discussed above, there were clear discrepancies in the collection times of wound fluid samples both between and within the same patients. This is likely to have a significant bearing on the quantification of the results. The type of acute wound fluid used in comparative studies with chronic wound is also important. Nwomeh et al. (1999) highlighted the limitations of using drainage bottles from acute surgical wounds in cutaneous wound healing research, since they demonstrated significant differences in MMP-1 and MMP-8 profiles in fluids from both mastectomy or myocutaneous flap reconstructive procedures compared to acute dermal wound fluid retrieved from under occlusive dressings covering full thickness biopsy sites. MMP activities were significantly more pronounced in the dermal wound fluid group. Therefore, in comparative cutaneous wound healing studies, caution is required taking AWF samples from sub-dermal or deep fascial drains as opposed to fluids taken from under acute cutaneous wounds, when compared with CWF samples from the skin (Nwomeh et al., 1999). The sensitive assays employed for identification and quantification may contribute to the observed variability and confounding results noted between studies, hence the importance to perform these tests in the same batches (Staiano-Coico et al., 2000).

A single sample of wound fluid does not give a useful indication of the influence of proteases present on the wound healing process; multiple collections need to be easily reproducible and validated within each collection model (Stacey and Trengove, 1999). Caution is required when comparing fluids between patients

with age-matched, sex-matched and site-matched variables taken into consideration (Stacey and Trengove, 1999). Different cell types secrete many of the same growth factors and cytokines at different times, and maybe with differing roles (Staiano-Coico et al., 2000).

There are also difficulties maintaining control over the variables that exist within the wound bed and the patient's condition might complicate studies on human wound fluids. There is still great difficulty in determining which factors present in wound fluids are causative to either active or inactive healing processes, or simply a reflection of that improved or impaired process.

It is also important to highlight that in many of these studies (some of which do lack sizeable numbers), there appeared to be significant patient-to-patient variability in protease activity and patterns of protease degradation in CWF (Bullen et al., 1995; Grinnell et al., 1992; Trengove et al., 1999; Wysocki et al., 1993; Yager et al., 1997). Grinnell and Zhu (1996) suggest that since the overall patterns of fibronectin degradation were similar for all the wound fluid samples taken from each patient, but very different from one patient to the next, the variability is with the ulcer itself and not the time at which the fluid sample is taken. Yager et al. (1997) also comment that any variability in protease activity noted in ulcer fluids, for example, may simply be a reflection of ulcers that are in different phases of healing or non-healing (Yager et al., 1997).

1.5 Summary of Literature Review

TNP therapy is the application of a sealed foam dressing into or onto a surface wound which is under the influence of topical subatmospheric pressure. TNP has been used in the treatment of a heterogeneous group of acute and chronic wounds over the last 10 years, but is less well described in the treatment of burns. An accepted foam dressing and controlled subatmospheric pressure machine (V.A.C.™) has been commercially available for 7 years.

Despite numerous case reports and larger clinical series, there has been a paucity of randomised controlled clinical trials to test the efficacy of TNP on wound healing. Large, properly randomised, inter-patient clinically controlled trials are currently in progress. Much of the difficulty in studying the effects of TNP in human cutaneous wound healing stems from the inherent problems surrounding the selective application of TNP therapy on an open, exudative wound surface, which up to now has not been achievable in the same patient. To establish and maintain negative pressure within the foam dressings the whole wound surface and surrounding dry skin needs to be sealed. This precludes the selective use of TNP therapy on adjacent wound sites in intra-patient controlled conditions when testing its efficacy in wound healing.

The mechanisms of action of TNP therapy in wound healing appear to be multifactorial. TNP increases blood flow and has been shown to improve skin flap survival experimentally. Granulation tissue formation in TNP-treated wounds is prolific. There is evidence for a reduction in bacterial colonisation in inoculated experimental wounds. An attenuation of interstitial fluid and the removal of substances (including proteins and inflammatory cells) from TNP-treated wounds have also been proposed following experimental conditions but has not been confirmed in human wounds. There is circumstantial evidence to support a mechanical role implicating TNP in wound healing. Despite these proposed mechanisms, there is very little objective evidence in the literature to directly support these claims and the significance of each of these individual factors remains undetermined. The role of TNP therapy in the treatment of burns has not been examined extensively and there are no published accounts of its effect on human burn injury.

Neutrophils are the most prominent of the inflammatory cells that enter wounds following injury to the skin. With the progression of local burn wound inflammation, local and systemic mediator production increases. The relative roles of these mediators both directly and through neutrophil-mediated events in normal and burn wound healing, remain to be fully determined.

Following burn injury, capillaries are progressively occluded in a zone emanating from the point of greatest trauma. Preceding this is a zone of reduced blood flow, which becomes static over a period of 24 to 48 hours. This zone of stasis is reversible and consequently the progressive dermal necrosis is potentially preventable. There is evidence that the migration of activated neutrophils into the zone of stasis of partial thickness burns may play an important role in the progression of dermal ischaemia. Blocking neutrophil access into burn tissue is associated with improvements in dermal vascular blood flow when observed in animal models. Neutrophils are capable of releasing massive amounts of oxidants, arachidonic acid metabolites and proteases; supporting the role of neutrophils as central mediators of progressive ischaemia noted in partial thickness burns.

Neutrophil-derived proteases have been implicated in the aetiology of difficult-to-heal wounds such as chronic wounds and some burn wounds. Evidence suggests that the most potent of these proteases might be NE. Elastase is implicated in a number of physiological processes in wound healing including the emigration of neutrophils into sites of tissue injury and the breakdown of extracellular matrix proteins to aid tissue repair. Pathological tissue destruction can occur when the proteolytic activities of NE by neutrophils is excessive, inappropriate or prolonged, although the mechanisms by which this is established are not fully understood. Endogenous inhibitors, the largest and most important of which is α_1 -PI, tightly controls elastase activity. Unregulated protease activity may mediate extracellular tissue damage in a number of acute and chronic inflammatory wound states, and may be caused by an imbalance between NE and α_1 -PI.

Wound fluid provides a valuable opportunity to understand the temporal activities of the extracellular environment of healing and poorly healing wounds. To examine the protease/anti-protease hypothesis more closely, investigators have measured the relative temporal ratios of proteases and their inhibitors in wound

fluid. A number of methods of collecting wound fluid have been described; the most suitable of which depends on the type of wound under investigation, the frequency and amount of fluid required. The current literature suggests that the levels and activities of proteases measured in wound fluids are elevated in both healing and non-healing wounds compared to normal skin. There is corroborative evidence from these studies that some MMPs and serine proteases may play a significant role. Furthermore, the majority of these active, elevated proteases are primarily, if not exclusively, of neutrophil origin.

The analysis of wound fluid collected from acute and chronic wounds is being increasingly used as an investigative tool in wound healing research. However, standardisation in the methods of wound fluid collection and storage is mandatory if correct temporal interpretation is to be made. Quantitative analysis of wound fluid parameters using intra-patient controlled studies may provide a more accurate means of addressing this problem.

2 HYPOTHESIS AND STUDY AIMS

Hypothesis: Topical Negative Pressure alters the acute wound environment

Four study chapters are presented in this thesis. The aims of each study chapter are laid out below.

Chapter 3: The design of a Topical Negative Pressure Device for selective subatmospheric pressure therapy on human dermal wounds

This study describes a new method of applying selective TNP to unwounded human skin and acute human dermal wounds. The application of two TNP designs is described, one of which can be utilised as a research tool to collect wound fluid. The second part of this chapter is concerned with validating this new model both in vitro and in vivo.

Chapter 4: Temporal analysis of Total Protein in wound fluid under the influence of TNP following partial thickness dermal injury

The 'irrigation' TNP device described is used on acute human donor site wounds and acute partial thickness burn wounds to assess the effects of TNP therapy on the constituents of wound fluid, measuring Total Protein as a marker.

Chapter 5: Histological investigation into the effects of Topical Negative Pressure therapy on neutrophils and their distribution in partial thickness dermal wounds

A histological study is conducted to determine whether TNP can directly influence neutrophil numbers and their distribution in acute dermal wounds by immunohistochemical comparisons of TNP-treated and control-treated sites.

Chapter 6: The influence of Topical Negative Pressure therapy on the temporal analysis of Neutrophil Elastase activity and α_1 Protease Inhibitor in wound fluid of human donor site and partial-thickness burn wounds

Neutrophil Elastase and its endogenous inhibitor, α_1 -PI, are assessed under the influence of intermittent TNP therapy. The aim is to demonstrate a relative temporal profile of both the protease activity and its inhibitor and to establish whether the protease/anti-protease ratio can be modulated by intermittent TNP therapy.

3 The design of a Topical Negative Pressure Device for selective subatmospheric pressure therapy on human dermal wounds

3.1 Introduction

The contribution of Topical Negative Pressure (TNP) to human wound healing has been demonstrated in a number of individual case reports and uncontrolled clinical series. An understanding of the mechanisms by which TNP facilitates wound healing remains unclear, yet the evidence suggests that this is likely to be multifactorial.

There is a paucity of published work describing the effects of TNP against concurrent patient controls. Human skin is unique and varies according to age, sex, race and region of the body. Some wounds are also heterogeneous in behaviour. To determine true differences in the effects of TNP, an ideal study would apply TNP therapy to part of the same wound with an adjacent area treated as a control.

The use of a sealed foam dressing on exudative acute and chronic wounds determines that the whole wound surface must be covered hermetically, to ensure that negative pressures are maintained. For this reason, there is no good method of testing TNP therapy against standard treatments, in intra-patient controlled human trials.

Hence, a new approach is necessary. To simplify this problem, a design construct is required that might allow one aspect of a wound surface to be treated with TNP independently of an adjacent site. In this way, the creation and maintenance of a discrete subatmospheric pressure system might allow comparable testing of TNP versus controls, and provide ways of exploring its mechanism(s) of action.

The aim of Study One was as follows:

1. To design a TNP device that could be applied selectively to an open, acute dermal wound surface.
2. To create within the design, an opportunity to frequently and reliably collect wound fluid using an irrigation system over a designated wound surface area
3. To validate the newly designed TNP devices and provide evidence for its efficacy
4. To test the safety of the new device on normal skin and acute wounds

3.2 Materials and Methods

Any novel construct, designed to provide TNP to a wound surface for subsequent studies, required certain criteria to be met (see Table 3).

Table 3 Criteria required in the design of a TNP device

1. A device that would mimic standard TNP therapy in the clinical setting
2. Manufactured easily on-site, within the hospital, at minimal cost
3. <i>Manufactured and stored, prior to its use in an elective or emergency setting</i>
4. Robust and reliable enough to allow subatmospheric pressure therapy over a 48-hour period
5. Used on, and surrounded by an exudative wound surface
6. Not move from its original site of application once the therapy is commenced
7. Not move from its original site of application despite turning the suction device off during therapy
8. Utilises standard VAC™ dressings, which are the most widely used and accepted clinically
9. Ensure sterility to the wound surface
10. <i>Physically safe and harmless to use on an open wound surface</i>
11. Not create discomfort over and above that tolerated using standard dressings in clinical practice

For the purposes of wound fluid analysis, further criteria would need to be met (see Table 4).

Table 4 Criteria required in the design of a TNP device for wound fluid collection

1. Allow for surface wound fluid collection within a specified TNP site under investigation
2. Allow for <i>reliable and frequent</i> wound fluid sampling over a 48-hour time period
3. A system that would not interfere with the post-operative day-to-day medical and nursing care
4. Retrieval of <i>fresh</i> wound fluid directly for immediate centrifugation and storage at -70°C

The following TNP devices were designed and constructed at the Stoke Mandeville Burns and Reconstructive Surgery Research Trust at Stoke Mandeville Hospital. Some of the materials used in the construction were adapted from

commercially available dressings manufactured by Kinetic Concepts, Inc. (KCI)¹, Dorset, UK. In order to measure the effects of TNP on normal or wounded skin, a system of sponge 'containment' was required. A foam dressing placed within a fixed outer wall can be established by using a ring design.

A 'standard' TNP device was designed to exactly mimic the normal application of the VAC™ dressings supplied commercially. An 'irrigation' TNP device was also designed to enable wound fluid collection.

3.2.1 The design of a standard TNP device

The first prototype ring created, utilised the top 1.5cm and expanded flange of a plastic beaker. A second prototype ring was made from the top 1.5cm of a 40mm and 60mm diameter Galipot™ (Warwick Sasco Ltd. UK). A 9.5mm diameter hole was drilled through the sides of the plastic rings and a protective polyvinyl chloride (PVC) grommet with an internal diameter of 6.0mm placed within it (Farnell Electronics, Ltd.). See Figure 1 on page 82.

Sterile, medical-grade, PUE foam of a standard thickness (3.2cm) can be cut into a circular shape to fit inside the rings.

An evacuation tube with drainage holes at the distal end (the same tubing available in the standard VAC™ dressing pack provided by KCI) is placed within the PUE foam and exits the ring device through the PVC grommet (see Figure 2 on page 82).

An adhesive transparent dressing (available in the VAC™ foam dressing packs) loosely seals the 'sponge-in-ring' construct, to mimic the standard application of TNP dressings to wounds. The evacuation tubing is attached to a VAC™ pump via the canister provided. Sub-atmospheric pressure, pre-set by the operator, is then regulated by the VAC™ pump as required. In this fashion, the sponge can

¹ All the work described in this thesis remained independent of KCI, Ltd.

collapse unhindered within the firm outer plastic ring, whilst under the influence of negative pressure.

A significant problem, created by the use of such a device, concerns the pressure effects imposed by the base of the ring directly onto the skin or wound surface. Whilst under sub-atmospheric pressure, a protective material was necessary to prevent pressure ischaemia caused by the base of the ring. In addition, the material used must also have sterile, semi-adherent properties that would ensure that a seal could be maintained throughout the duration of a study (up to 2 days). Furthermore, it was important to make sure that the whole device could be removed as necessary for observations or measurements of the wound surface as required.

Tested with the device on normal skin, the first protective material used was Varihesive™ Wafers (Convatec Ltd., UK.); a hydrocolloid product designed for use in stoma care. 100x100mm sterile wafers can be cut to fit the base of the prototype ring designs described, and is shown in Figure 3 on page 82.

The protection afforded by the hydrocolloid dressing was shown in testing, both on normal skin and subsequently on acute dermal wounds, to fail over a period of 12 hours. Firstly, as the hydrocolloid takes up fluid, it swells and distorts under the application of sub-atmospheric pressure. The base of the ring becomes visible and pressure marks become apparent on the skin surface (see Figure 17 on page 94). The second difficulty was that the TNP device could not be removed from the surface of the wound for inspection without disrupting the hydrocolloid base. Thirdly, as the TNP device was to be adapted later to include an irrigation port for wound fluid collection (see section 3.2.2), the collection of fluid samples would likely to be contaminated with proteins present in the material (including gelatins) from the dissolving hydrocolloid base padding.

Figure 1 Prototype ring designs with holes and PVC grommets in situ

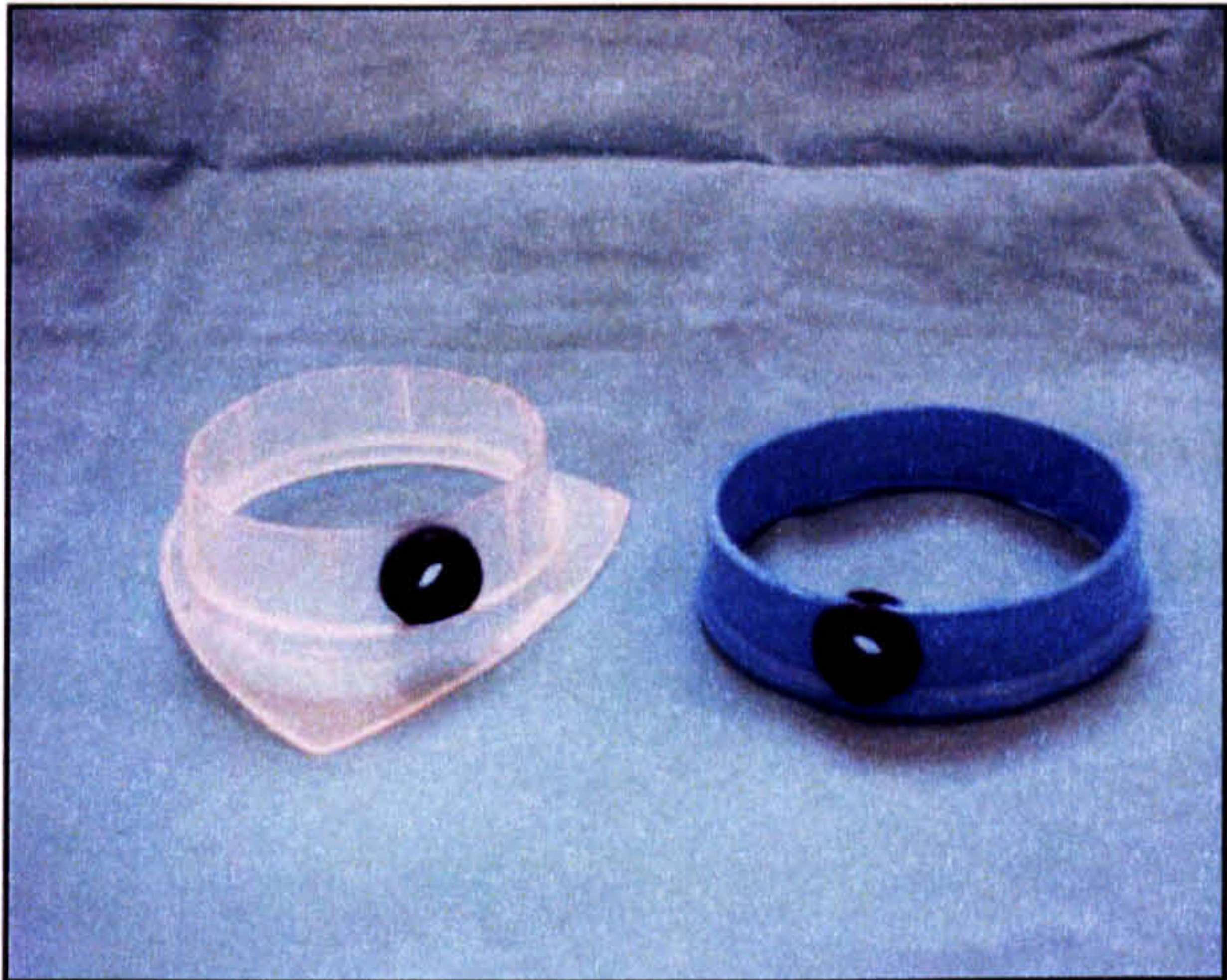


Figure 2 Polyurethane foam is cut to fit inside the ring device, the evacuation tubing placed within the foam which exits through the ring as shown

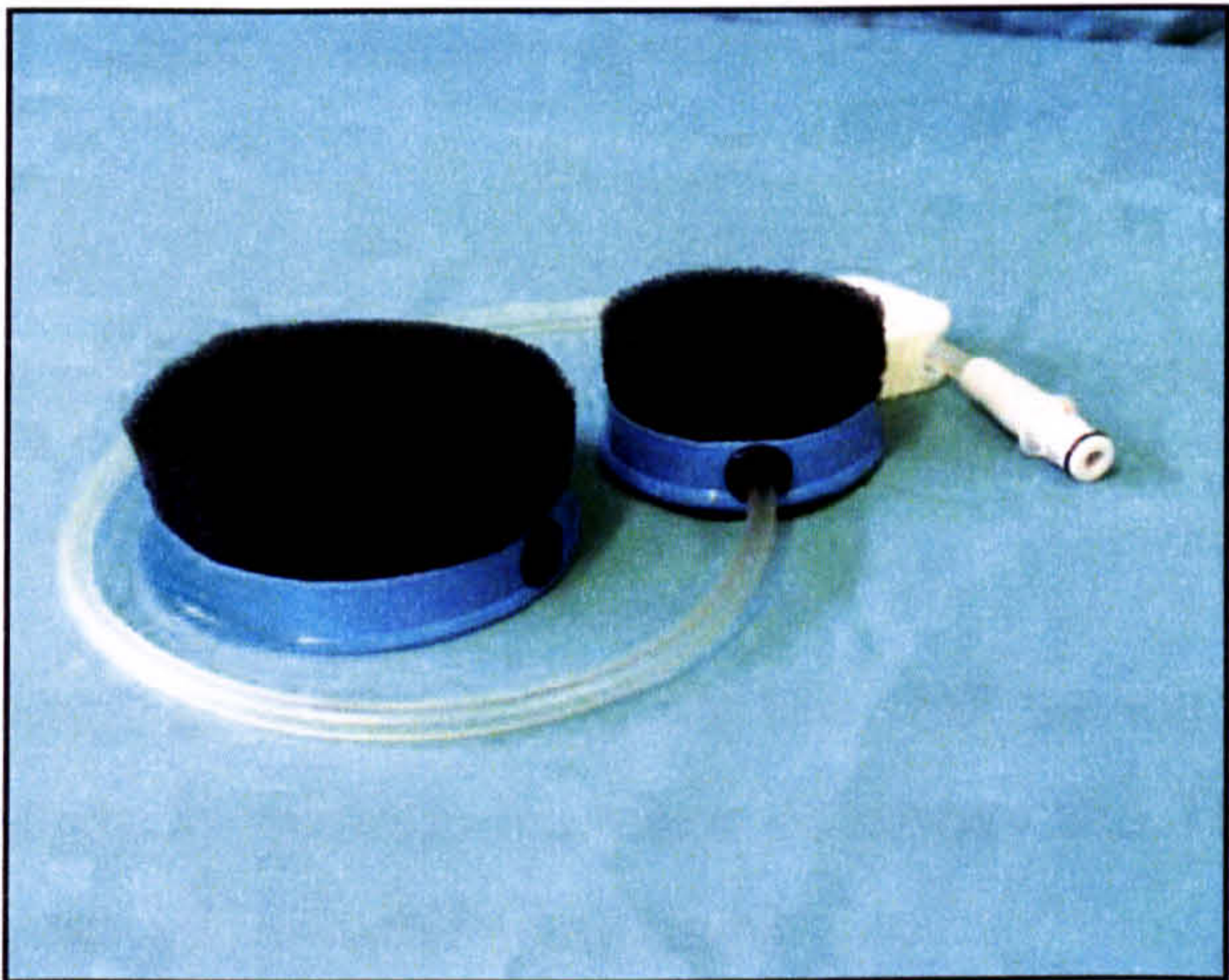
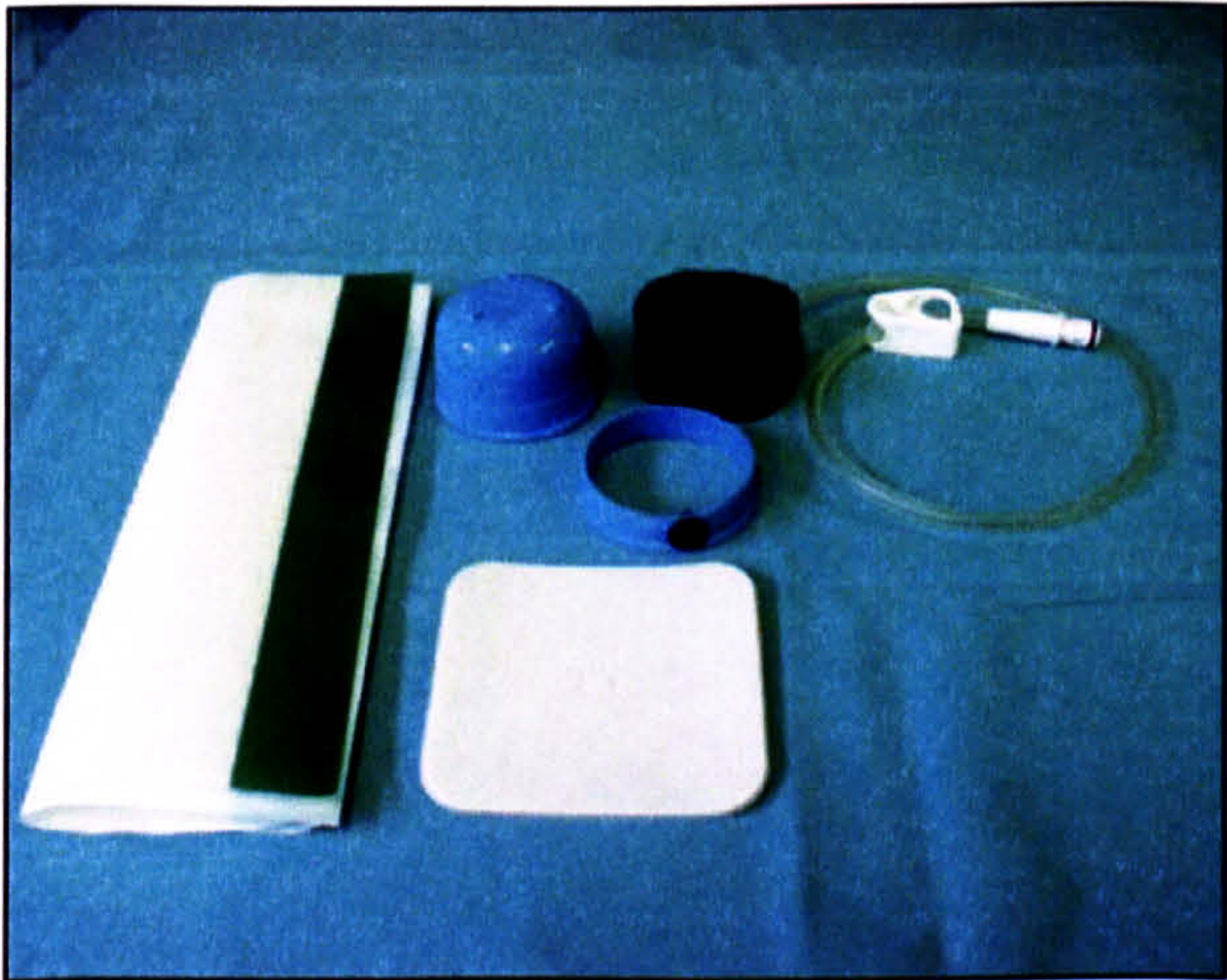


Figure 3 Construction of TNP device with a hydrocolloid base dressing



The above problems concerning protection to the skin during TNP therapy were overcome by the use of a sterile flexible polymer sheet (Dermal Pad™, Spenco Healthcare International Ltd.) The 10cm x 10cm sheet with a thickness of 3mm was glued directly onto the base of the Galipot™ ring design using Loctite™ adhesive glue. A hole was then cut from inside the ring (see Figure 5 on page 85). The TNP device was constructed in the same fashion with the outer adhesive transparent dressing sealed to the blue ring itself.

This new protective base dressing conferred the following advantages.

- A. The 'tacky' quality of the dermal pad allowed for adherence to the skin or wound without a direct adhesive nature to it. A film of fluid between wound and dermal pad also helped to establish a seal if necessary
- B. The Dermal Pad™ dressing does not distort or breakdown
- C. It would now be possible for the TNP device to be removed at any stage of a study to inspect, measure or biopsy the TNP-treated wound. The same device could then be replaced

An example of the final TNP design is shown in Figure 4 below and Figure 6 on page 85. Testing on normal skin and a number of different acute wound surfaces demonstrated that the most suitable device involved the 40mm and 60mm diameter galipot™ ring systems.

Figure 4 Schematic diagram of standard TNP device

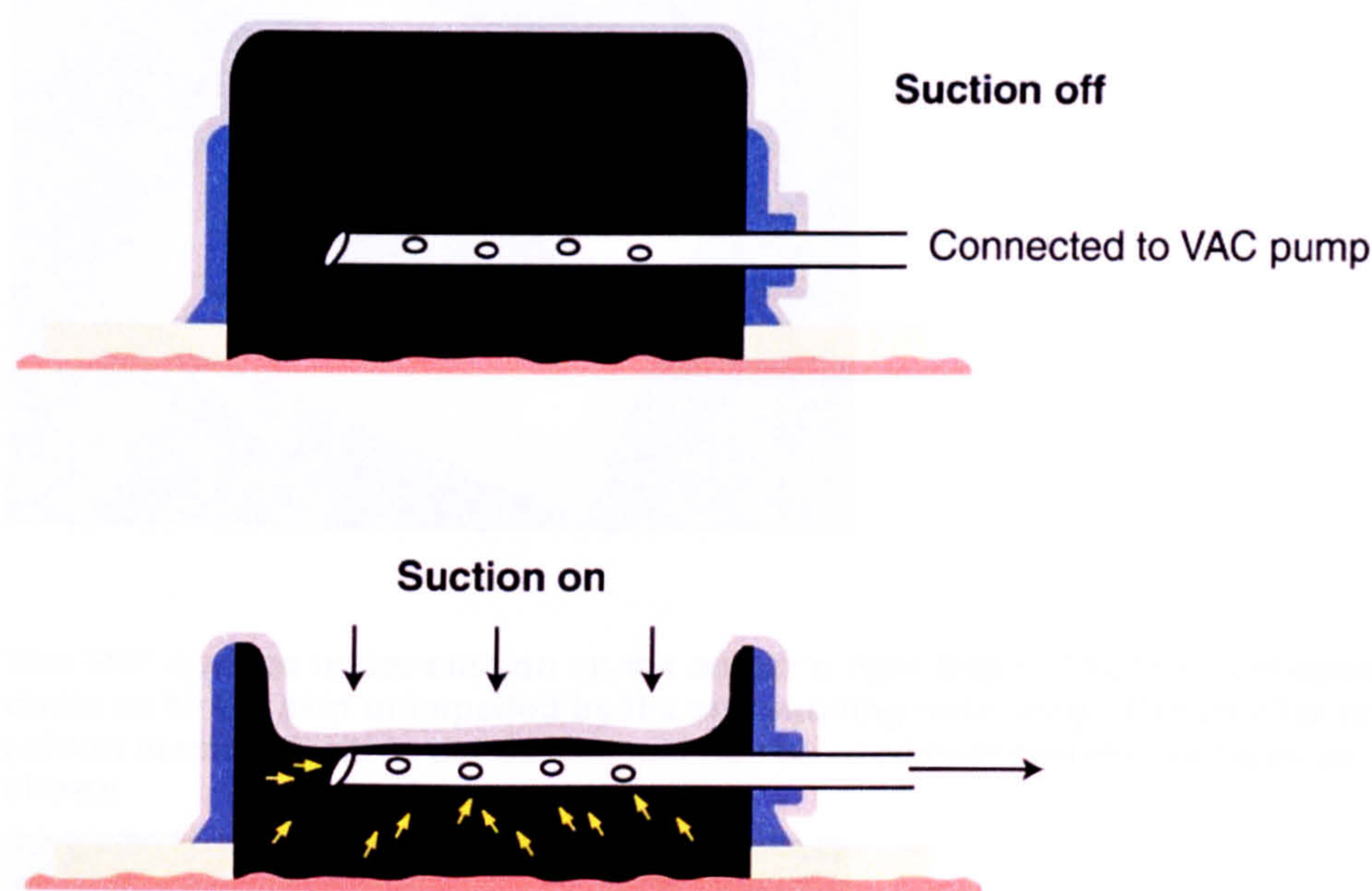


Figure 5 A 40mm diameter plastic ring is glued to the polymer pad and a hole cut within it

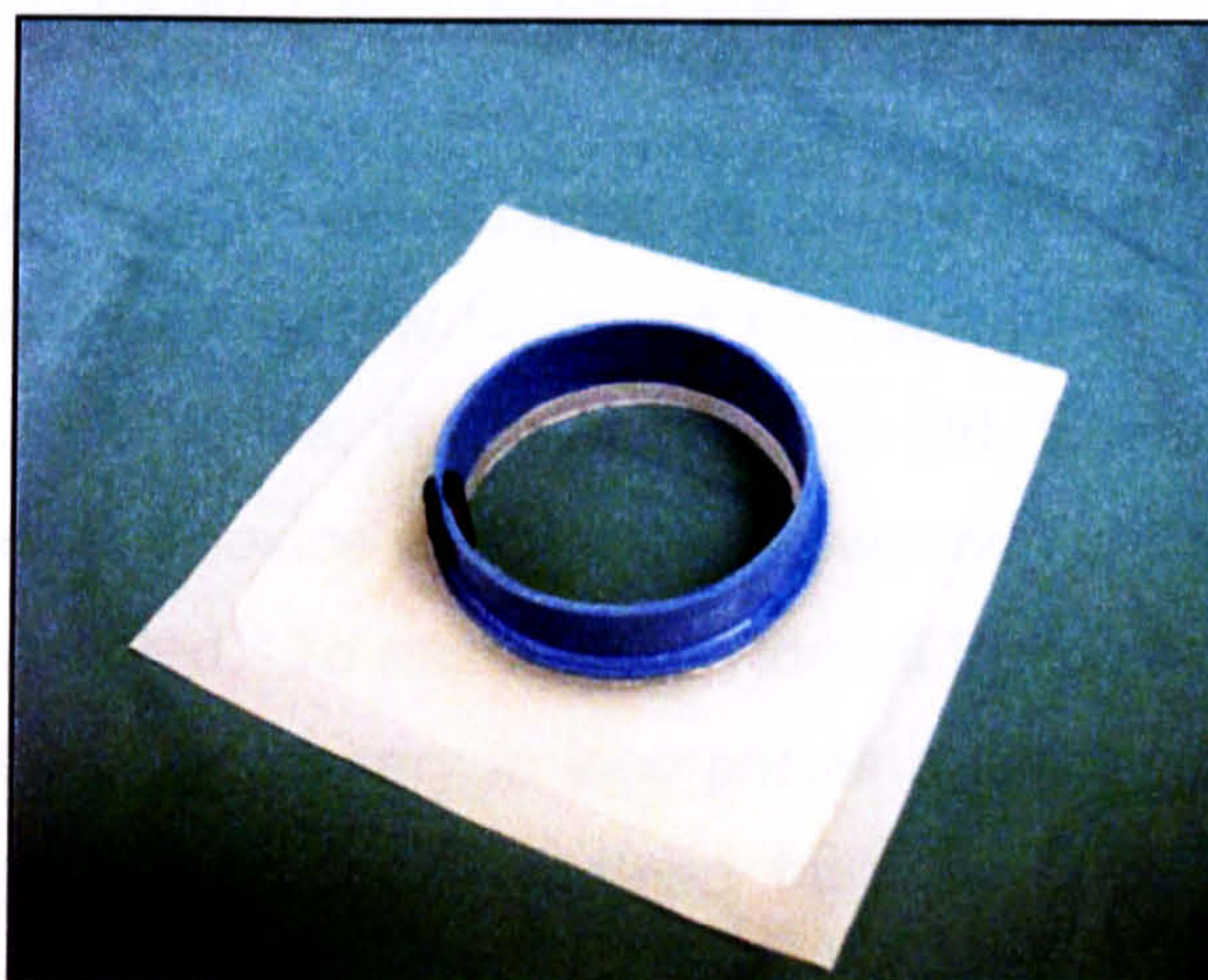


Figure 6 The TNP devices under suction on the author's right thigh. The foam collapses down on to the skin unimpeded by the surrounding outer ring. The smaller ring (40mm diameter) within the dermal pad can be used over convex surfaces as shown



Figure 7 Irrigation tubing, with holes in the tubing, within the lower chamber of the irrigation TNP device



3.2.2 The design of an irrigation TNP device for fluid irrigation and collection

The above design was adapted to enable the collection and analysis of wound fluid regularly every few hours as necessary. Normal Saline irrigant fluid (0.9% w/v Sodium Chloride, B. Braun Medical Ltd, Sheffield, UK.) was instilled into the foam within the device and the diluted wound fluid present within the system could then be collected. To ensure that Normal Saline bathed the surface of the wound *directly*, it was necessary to incorporate a lower circular foam chamber (adjacent to the wound surface), separated from an upper circular foam chamber by a perforated film barrier. The purpose of a perforated barrier was to contain the fluid for a specified period at the wound surface before being sucked away under the influence of negative pressure.

A TNP ring and polymer pad was constructed as described above. Irrigation tubing with holes along the distal 3.5cm was placed within the ring through the PVC grommet (Figure 7 on page 85).

A 1.6cm thick circular piece of PUE foam was cut to fit inside the ring. A 6cm x 7cm transparent film dressing (Tegaderm™, 3M Healthcare, Canada) was placed over the foam and sealed to the blue outer ring. Four 1.5cm perforations were made in the transparent dressing 0.5cm from the edge of the ring (as shown in Figure 8 on page 87). Placement of these perforations peripherally was performed to ensure that the fluid instilled into the lower chamber had to traverse the wound surface before being taken up by the foam in the upper chamber above.

Circular PUE foam of a standard thickness (which was also used in the standard device described in section 3.2.1) was placed over the lower chamber and an evacuation tube, with similar distally based holes, positioned within it. The upper chamber and evacuation tubing was sealed as before (Figure 9 on page 87).

After application of 125mmHg subatmospheric pressure to the upper evacuation tubing, the upper circular foam (in continuity with the lower foam) collapses down onto the skin. The dermal pad itself is not directly under the influence of suction (Figure 10).

Figure 8 Construction of the lower chamber using 1.6cm thick foam and a transparent dressing sealed to the edges of the ring. Note the 1.5cm perforations at the periphery of the Tegaderm™ dressing

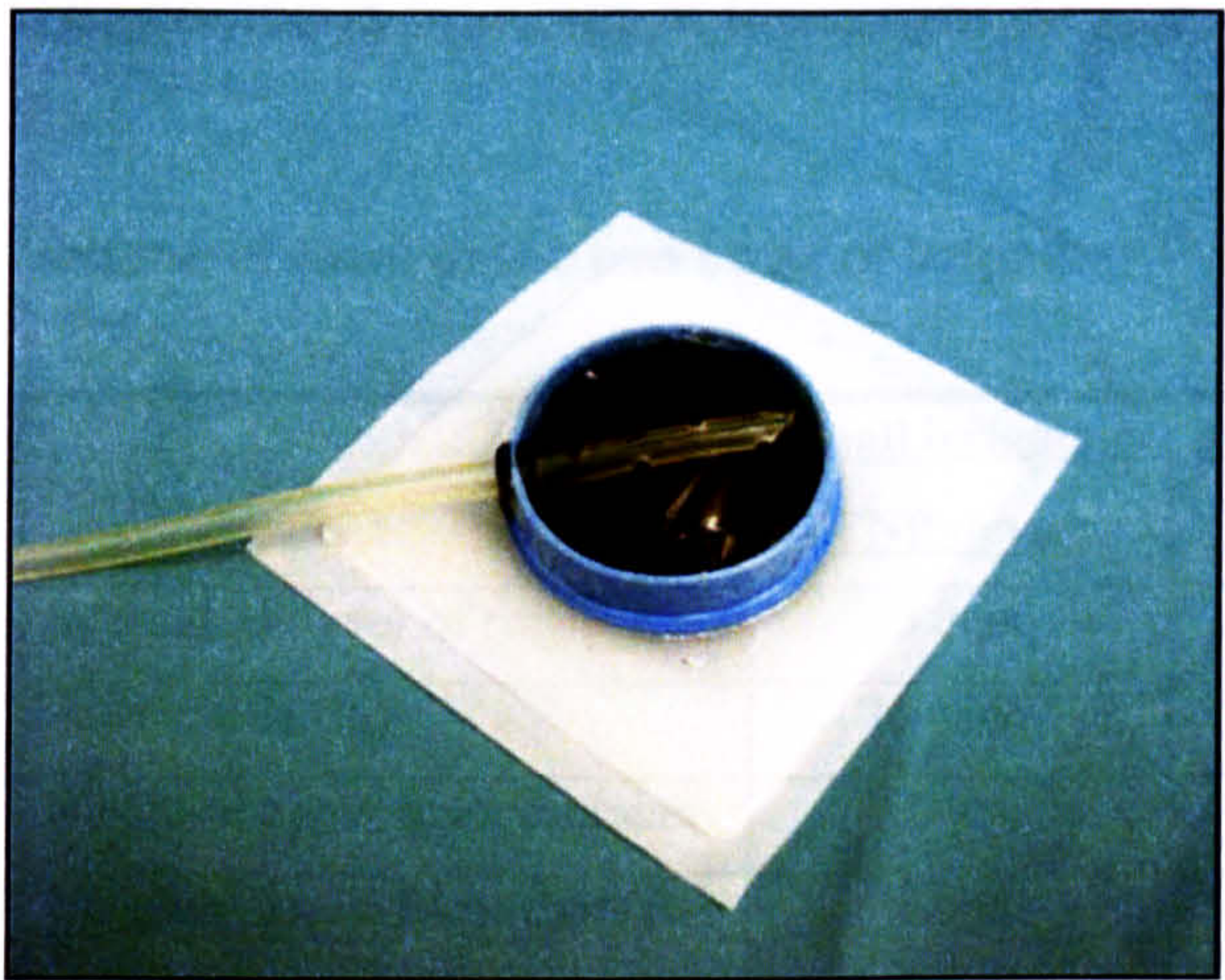


Figure 9 A TNP irrigation device showing the irrigation tubing entering through the ring (right) and the evacuation tubing exiting from the sealed upper foam (left)

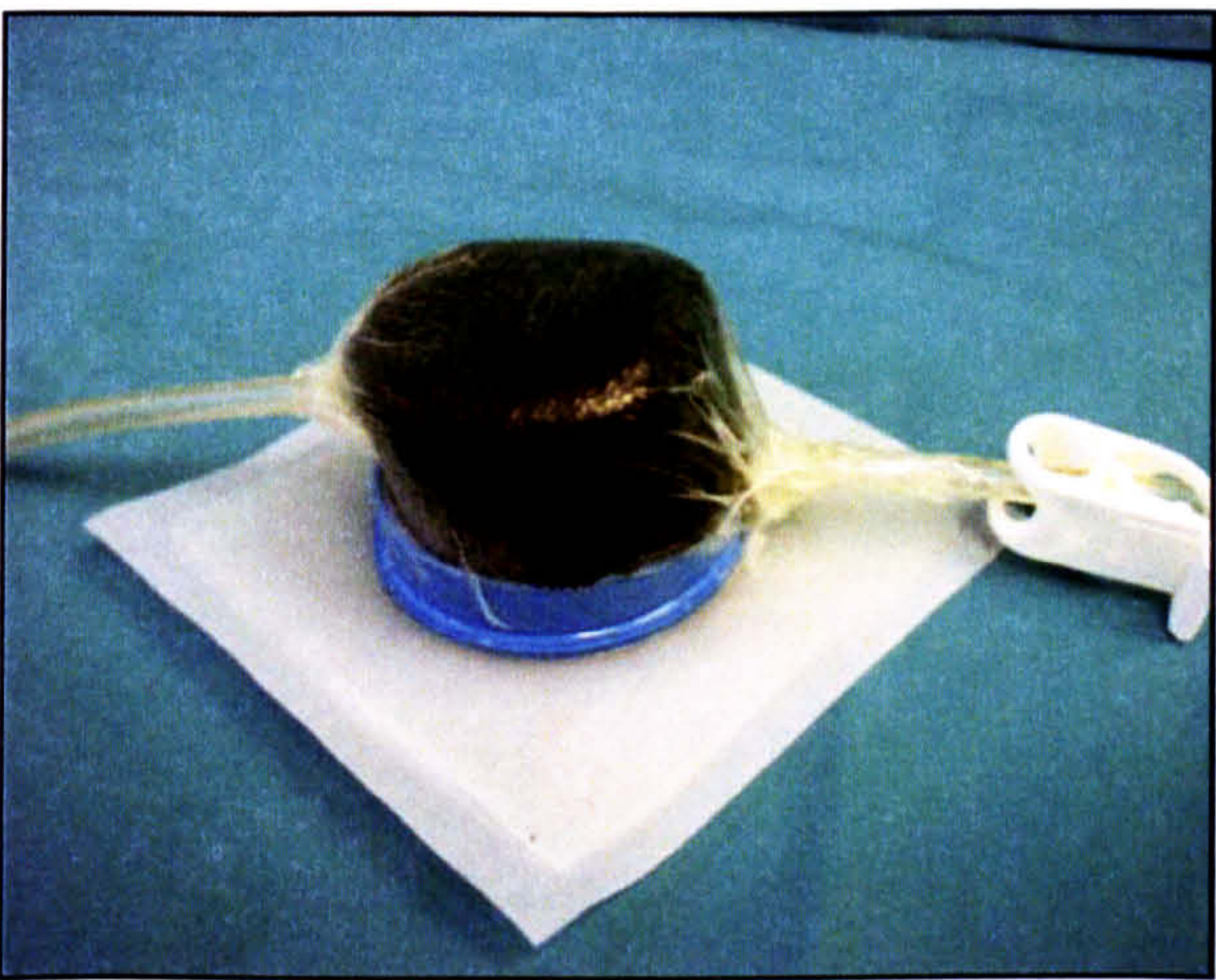
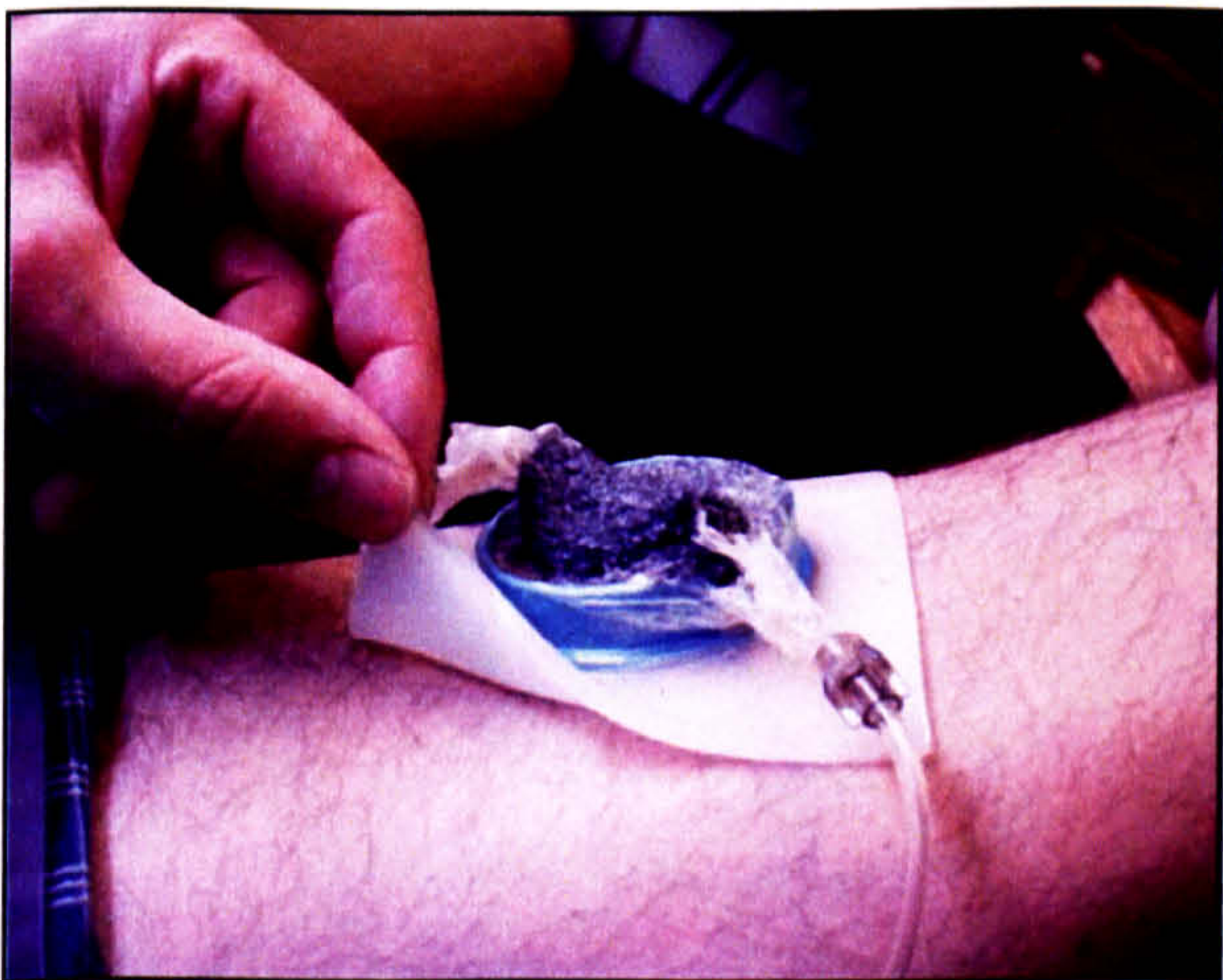


Figure 10 The irrigation TNP device under 125mmHg negative pressure on normal skin. The dermal pad is not under the direct influence of suction

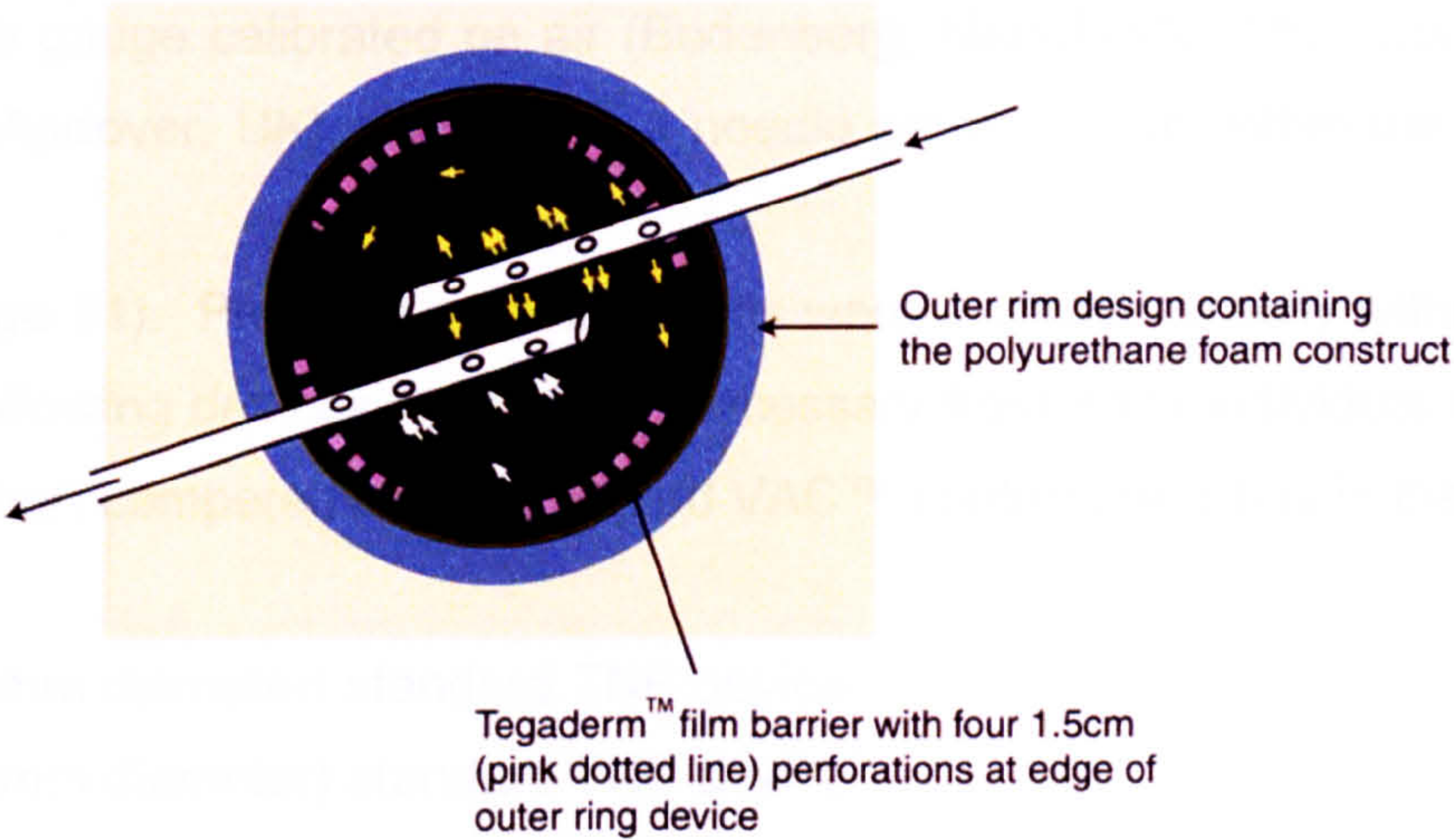
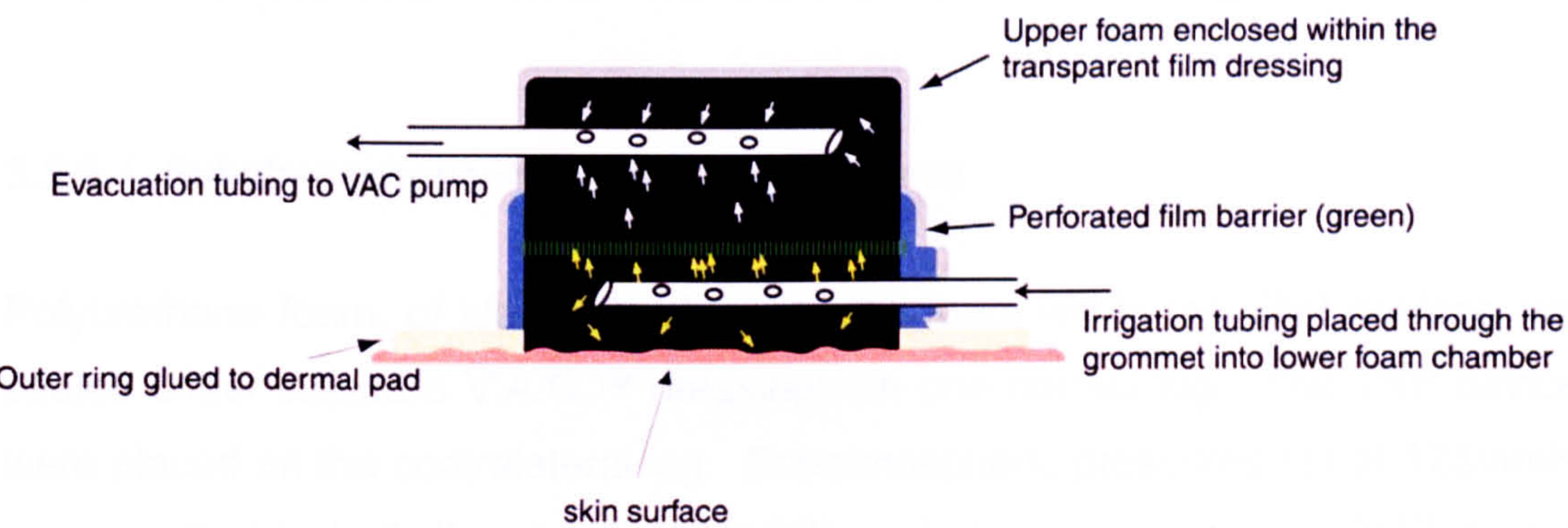


The volume of the foam in both the small and large irrigation TNP devices were calculated and displayed in Table 5. The volume of foam used in the small irrigation TNP device is half that of the larger device.

Table 5 Volume of foam in the two sizes of irrigation TNP device

	<u>Small irrigation</u> <u>TNP device</u>	<u>Large irrigation</u> <u>TNP device</u>
Internal diameter of ring	5.8cm	8.2cm
Area within ring	26.42cm ²	52.81cm ²
Height of upper foam chamber	3.2cm	3.2cm
Height of lower foam chamber	1.6cm	1.6cm
Total height of foam	4.8cm	4.8cm
Volume of foam	127cm ³	254cm ³

Figure 11 Annotated schematic drawing of the irrigation TNP device.



3.2.3 Validation studies on the TNP devices

The following studies were performed on normal skin or on the laboratory bench to validate their use as alternatives to the use of V.A.C.™ dressings.

3.2.3.1 Subatmospheric Pressure Measurements

Polyurethane foam, of identical volume to the foam within the TNP devices, was sealed under standard V.A.C.™ dressings on one normal leg. The TNP devices were placed on the contralateral leg. Subatmospheric pressures set at 125mmHg were applied to both the standard VAC™ sealed dressing and the TNP devices (see Figure 12 on page 91). Subatmospheric pressure readings were established using a pressure gauge calibrated on air (Budenberg, Manchester UK. Courtesy of Bioquell Plc, Andover, UK) attached to a needle probe placed within the foam (see

Figure 13 on page 91). Pressure measurements were recorded directly within the foam from the following devices (and where necessary from each individual upper and lower chamber) compared to the standard VAC™ control dressings in the:

1. Small (40mm diameter) standard TNP device
2. Large (60mm diameter) standard TNP device
3. Small Irrigation TNP device
4. Large irrigation TNP device

Figure 12 Testing the small TNP device against the standard VAC™ dressing technique

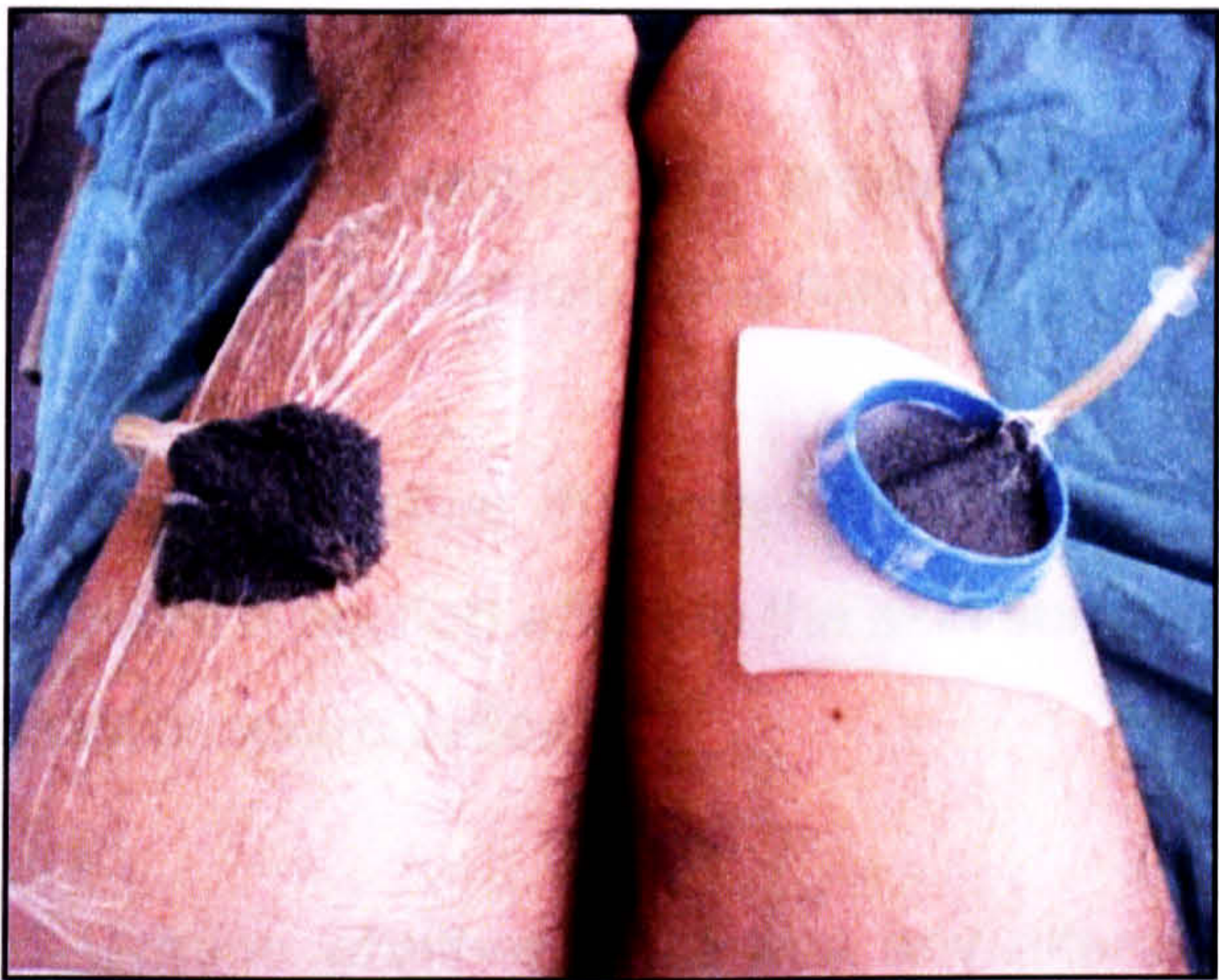


Figure 13 Needle probe placed within the upper chamber of the small TNP device whilst under negative pressure of 125mmHg

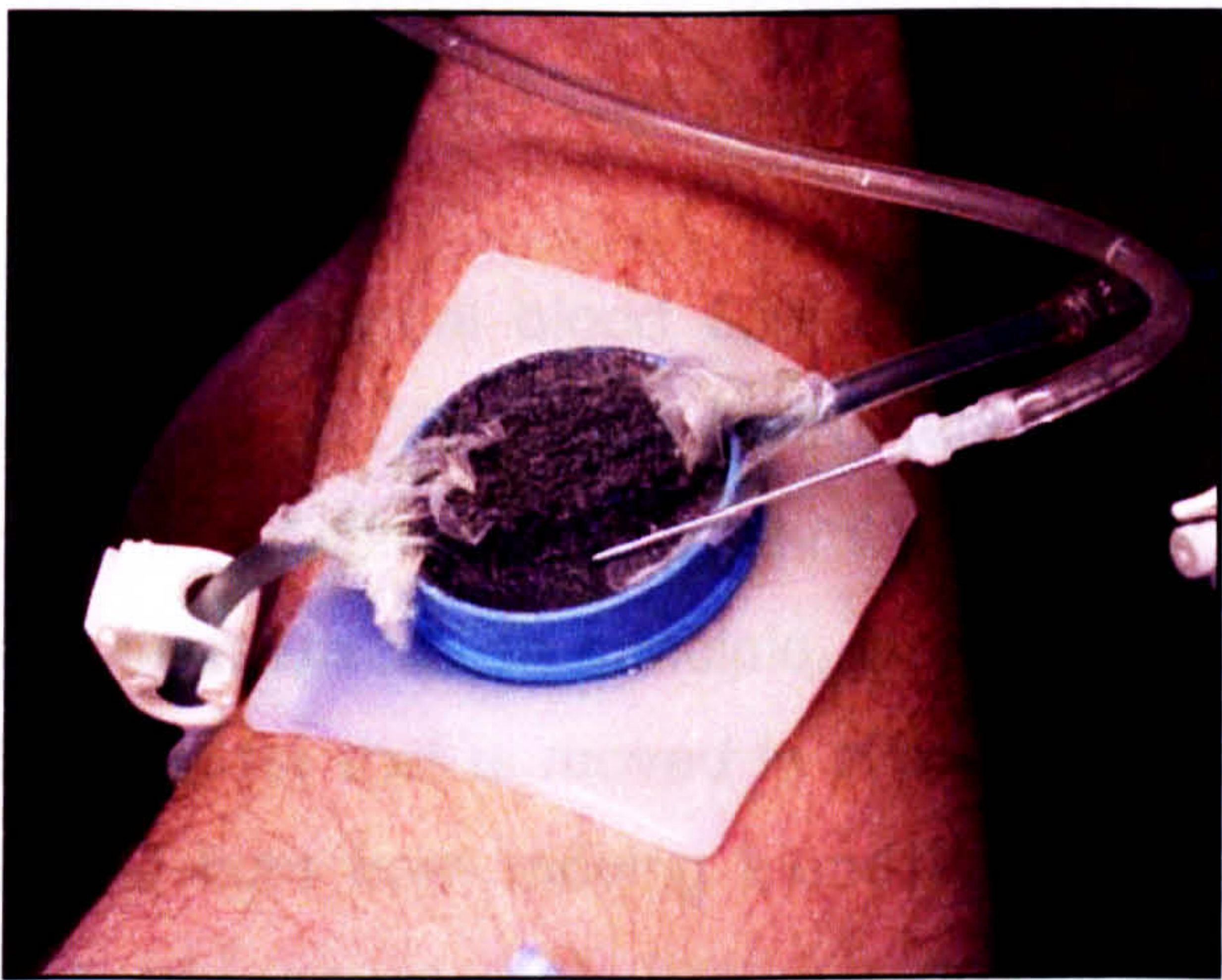
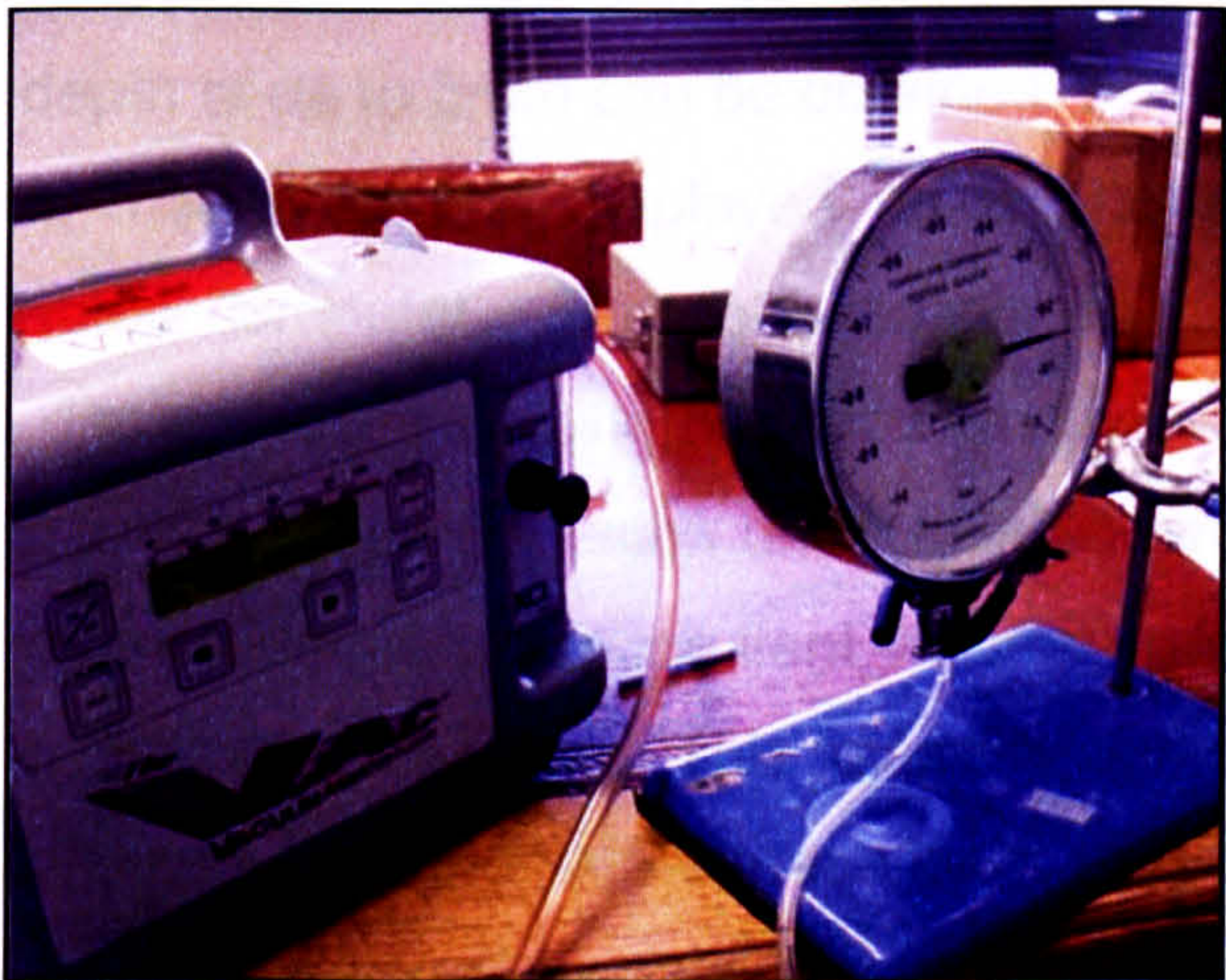


Figure 14 The pressure gauge at -0.16bar with the VAC™ pump set at a subatmospheric pressure of 125mmHg



3.2.3.2 Dermal blood flow studies of normal skin measured using laser Doppler

The assessment of subatmospheric pressure on dermal blood flow using the TNP device was required for the following reasons:

1. to establish whether the lower foam in the TNP irrigation device uniformly distributed a suction effect to the skin surface, by assessing spatial changes in skin microcirculation
2. to determine whether there was any alteration in dermal blood flow under the rim of the ring with or without the use of a protective dressing
3. to determine whether the effects of TNP therapy on dermal blood flow extended beyond the TNP device

Measurements of dermal blood flow can be achieved by using Laser Doppler Perfusion Imaging (LDPI, Moor Instruments, Ltd., Axminster, UK). The laser Doppler scanner comprises a 2-mW helium-neon laser mounted behind photodiodes and lenses. In principle, laser light is directed onto tissues such as the skin by a mirror that is moved in a controlled fashion using a stepper motor. Back-scattered light from moving blood and surrounding tissues is detected by photodiodes, and processed to produce images and values of perfusion using the Doppler principle. From this, blood flow can be measured in terms of flux (a quantity proportional to the product of the velocity of blood cells and their number concentration). The values can be expressed in arbitrary 'perfusion units'. A scanning depth of up to 3mm can be obtained.

The flux is measured and displayed as a 6-colour scale image directly on a portable laptop computer. The coloured pixels correlate to the blood flux (flow) within the dermis; red indicating high flow and blue, low flow. Purpose-built LDPI software provides mean perfusion units +/- standard deviation (SD). Additionally, the LDPI also produces a concomitant 16-scale grey 'photographic' DC image of the area undergoing scanning. This image is derived from the unprocessed light intensity reflected from the skin surface back to the photodetector. The DC and flux image is matched pixel for pixel that provides an accurate anatomical map.

3.2.3.3 Bonney’s Blue dye testing

The irrigation TNP device as described in Figure 11 on page 89, was designed so that irrigant fluid can be instilled into the lower foam chamber and after a specified period, the fluid can be drawn away into the upper foam chamber via the four 1.5cm perforated cuts made in the Tegaderm™ film dressing, whilst under suction. The following test using Bonney’s blue dye (Man Med, Bucks, UK) was performed for the following reasons:

- 1. to determine the distribution of blue dye over the surface of the skin
- 2. to establish whether the blue dye is seen to extravasate beyond the boundaries of the ring design

Bonney’s blue dye tests were performed on both the large and small irrigation TNP devices using both the hydrocolloid base and Spenco™ dermal pad designs. The protocol used in all of these tests is shown in Table 6.

Table 6 Protocol for dye testing in irrigation TNP device

1.	Bonney’s blue dye was diluted in Normal Saline (1:4 dilution)
2.	2mls of the blue irrigant fluid was instilled via a three-way tap into the irrigation tube with the VAC™ pump switched off and the foam normally expanded
3.	2mls of air was instilled into the lower irrigation tube over 5 seconds to ensure that all of the fluid was within the lower chamber and not lying in the irrigation tubing
4.	A sterile universal container (Sterilin, Ltd., UK) was attached to the upper evacuation tube, which was connected to the VAC™ pump
5.	After 1 minute, the VAC™ pump was switched on, the foam dressings collapsed and the blue dye was collected within the universal container
6.	The device was switched off and the foam dressings expanded again to the resting position
7.	The procedure was repeated every 2 minutes up to 10 occasions before the device was removed from the skin and photographs taken

Figure 15 Large TNP irrigation device with hydrocolloid base on a granuflex™ dressing. (a) suction off and (b) suction on. Note the collapse of foam chambers and the blue dye collecting in the fluid trap when TNP therapy is commenced

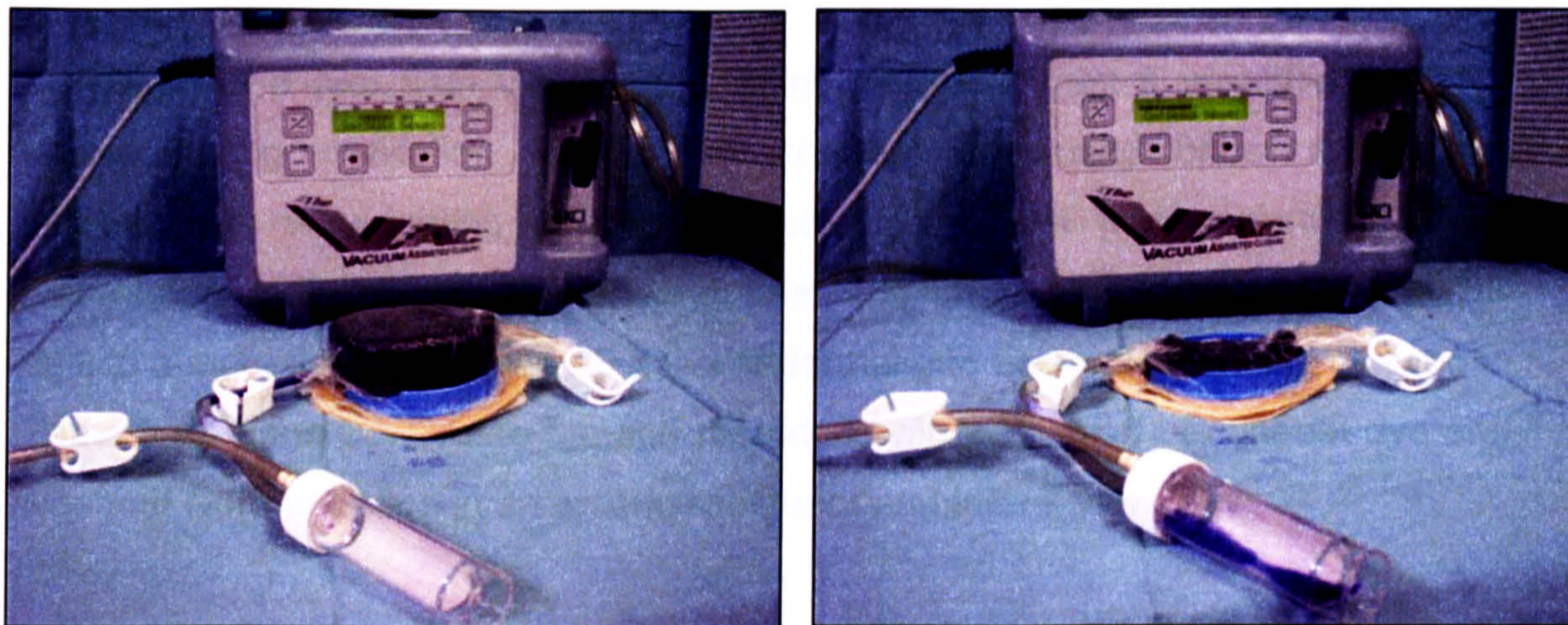


Figure 16 Large irrigation TNP device with hydrocolloid base under suction on author's lateral right thigh with Bonney's blue testing. Note the red laser dot on the upper foam that is in position for scanning from the LDI above (not shown).

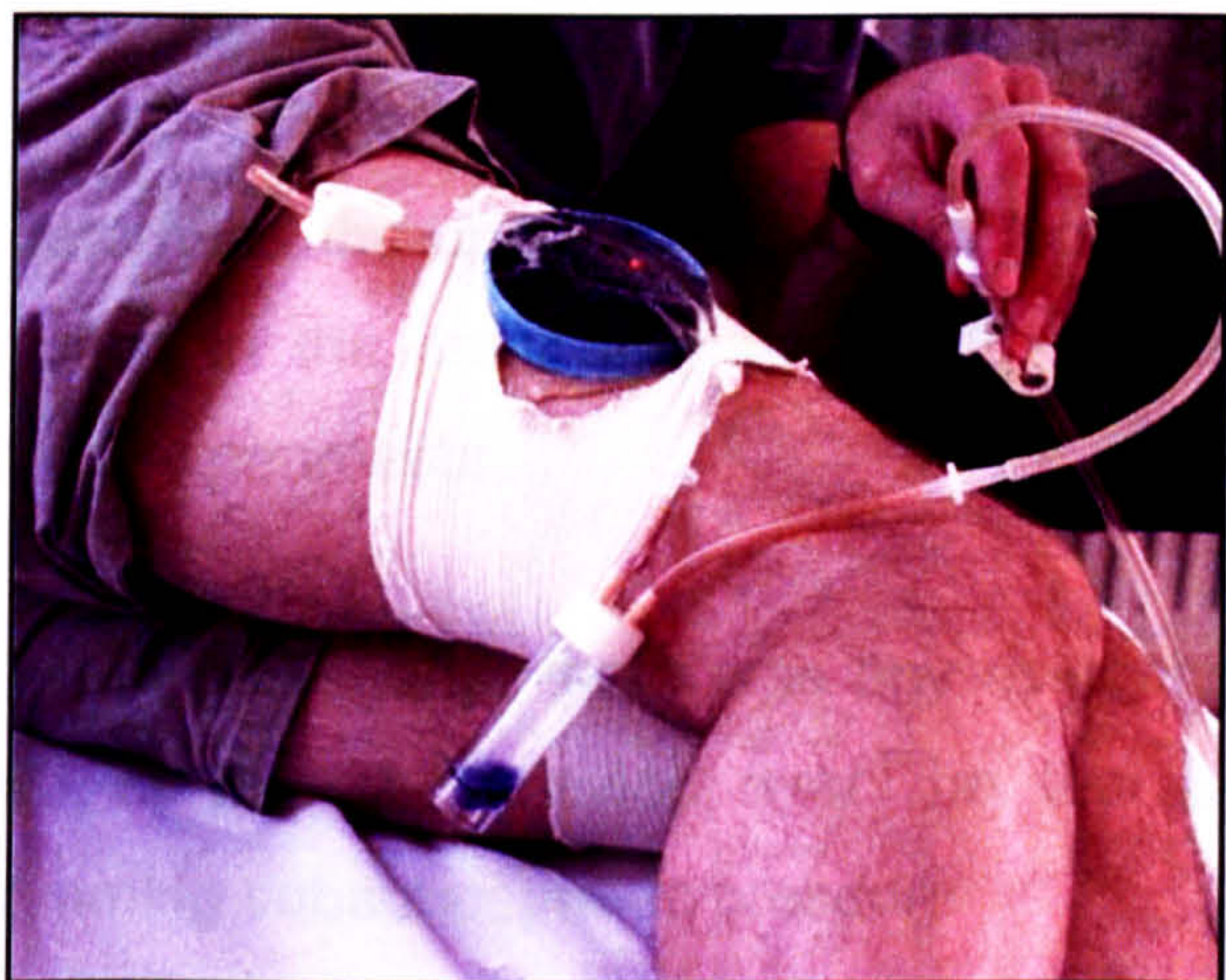


Figure 17 Removal of small irrigation TNP device following dye testing at 120minutes. Note the visible (now unprotected) blue rim of the device and the skin imprint.



3.2.3.4 Radiopaque dye testing

To establish a real time view of the distribution of irrigant fluid within the TNP device during instillation, Urograffin® contrast was used under radiological imaging. In vitro testing of the irrigation TNP device was performed in the Department of Radiology, Stoke Mandeville Hospital on both large and small TNP device attached to a Granuflex™ base or dermal pad using the protocol outlined in Table 6 on page 93. Video footage recorded both instillation of contrast dye and washout Normal Saline in an anterior-posterior and lateral view.

3.2.3.5 Fluid volume measurements on normal skin

The following tests described below were performed on dry, normal skin for the following reasons:

1. to determine the optimum volume of Normal Saline irrigant fluid that could be used in the small irrigation TNP devices for use during subsequent clinical experiments
2. to establish whether there was any evidence of pooling of fluid within the foam of the TNP device over the test period, despite application of 125mmHg subatmospheric pressure

Using the small irrigation TNP device, a number of tests were performed to determine the volume of irrigant fluid that could be used to ensure that fluid could be reliably retrieved through the system.

Three irrigant fluid tests were performed every ten minutes for up to 2 hours each using a different small TNP device. 2mls of Normal Saline was instilled through each device as described in the Bonney's Blue dye tests (see Table 6). The volume of irrigant fluid collected over the first minute was recorded; followed by the volume collected over the subsequent 9 minutes. These tests were performed on different days.

3.2.4 Testing the safety of the new devices on normal skin and acute wounds

The above validation tests on unwounded skin, using both the standard and irrigation TNP devices, were evaluated for safety and compliance. Testing the effects of the irrigation devices on open wounds in a clinical setting was carried out in conjunction with subsequent studies described later in this thesis. The patients described in section 4.2.2 were seen at variable times following their wound treatment with TNP therapy. The wounds studied included the acute donor site wound and partial thickness burn wound.

It was important to evaluate in these patients:

1. the effects of the TNP device on wounds undergoing TNP treatment
2. the safe use of the TNP device on acute wound surfaces
3. the risk of wound healing complications
4. patient compliance

3.3 Results

3.3.1 Standard TNP device

The design of the standard TNP device has been described. The validation of this device is documented below. This device was not used further in this thesis.

3.3.2 Irrigation TNP device

The irrigation TNP device has been described and further evaluated in the validation tests documented below. This device is used in the following chapters of this thesis.

3.3.3 Validation studies on the TNP devices

3.3.3.1 Subatmospheric Pressure Measurements

Pressure readings taken during these tests gave identical results of $-0.16\text{bar} \pm 0.02\text{bar}$ (which equates to a subatmospheric reading of approximately 120mmHg). There was no evidence of pressure differences between the standard VAC™ dressings and TNP devices. Furthermore, there were no differences between the different sized TNP devices, or between the lower and upper chambers within the irrigation TNP devices.

3.3.3.2 Dermal blood flow studies of normal skin measured using Laser Doppler

The scanned laser Doppler image of the skin surface is presented along side a concomitant photographic image of the same area.

The results of initial laser Doppler tests of a standard TNP device with *no* protective skin padding or barrier, was scanned as shown in Figure 18 on page 99. The flux image shows a circle of high flux (red colour) corresponding to the base of the unprotected rim, as noted in the corresponding photographic view. Furthermore, in the centre of the suction, patches of green and blue identify an area of lower flux. This suggests that the pressure effects imposed by the base of the outer ring device may influence the dermal blood flow within the device as well as directly under the unprotected rim.

The results of LDPI scanning of the first standard prototype device using the hydrocolloid dressing show that the hydrocolloid pad provided some protection to the skin, in that a mild 'halo' effect was seen on the flux image after 1 hour of TNP therapy (see Figure 19 and Figure 20 on page 99). The photographic image in Figure 20 shows the circle of normal skin directly under the influence of TNP as a slightly raised area. The corresponding flux image demonstrates that the area under the hydrocolloid dressing may also be influenced by TNP therapy as shown by the red, yellow and green colours.

Results using the small standard TNP device and small irrigation TNP device with the Spenco™ dermal pad as rim protection are shown in Figure 21 and Figure 22 respectively. A uniform circle of enhanced flux can be recorded which corresponds to the area of foam contact in both the devices tested with no evidence of a 'halo' effect seen, even after 5 hours of suction. The distribution of flux extends a few millimetres beyond the ring boundary when compared to its corresponding photographic image taken at the same time using the LDPI.

Figure 18 DC and flux image of author's left sided chest after 5 minutes of TNP therapy at 125mmHg using the small blue ring device without skin pressure protection (scan distance: 32cm)

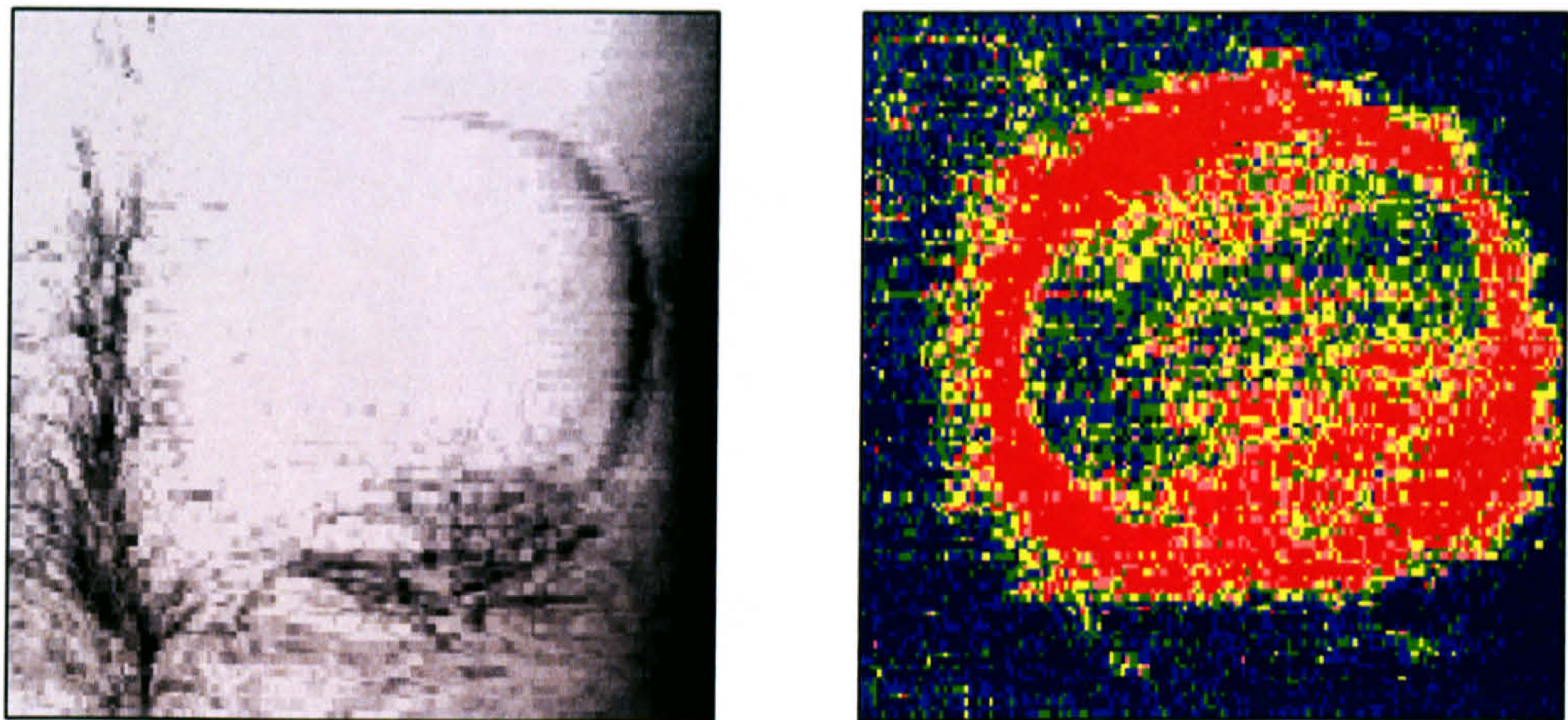


Figure 19 DC and flux image of normal left thigh skin before application of small standard TNP device. The black marker pen marks the outer boundary of the hydrocolloid protective dermal pad (scan distance: 37cm)

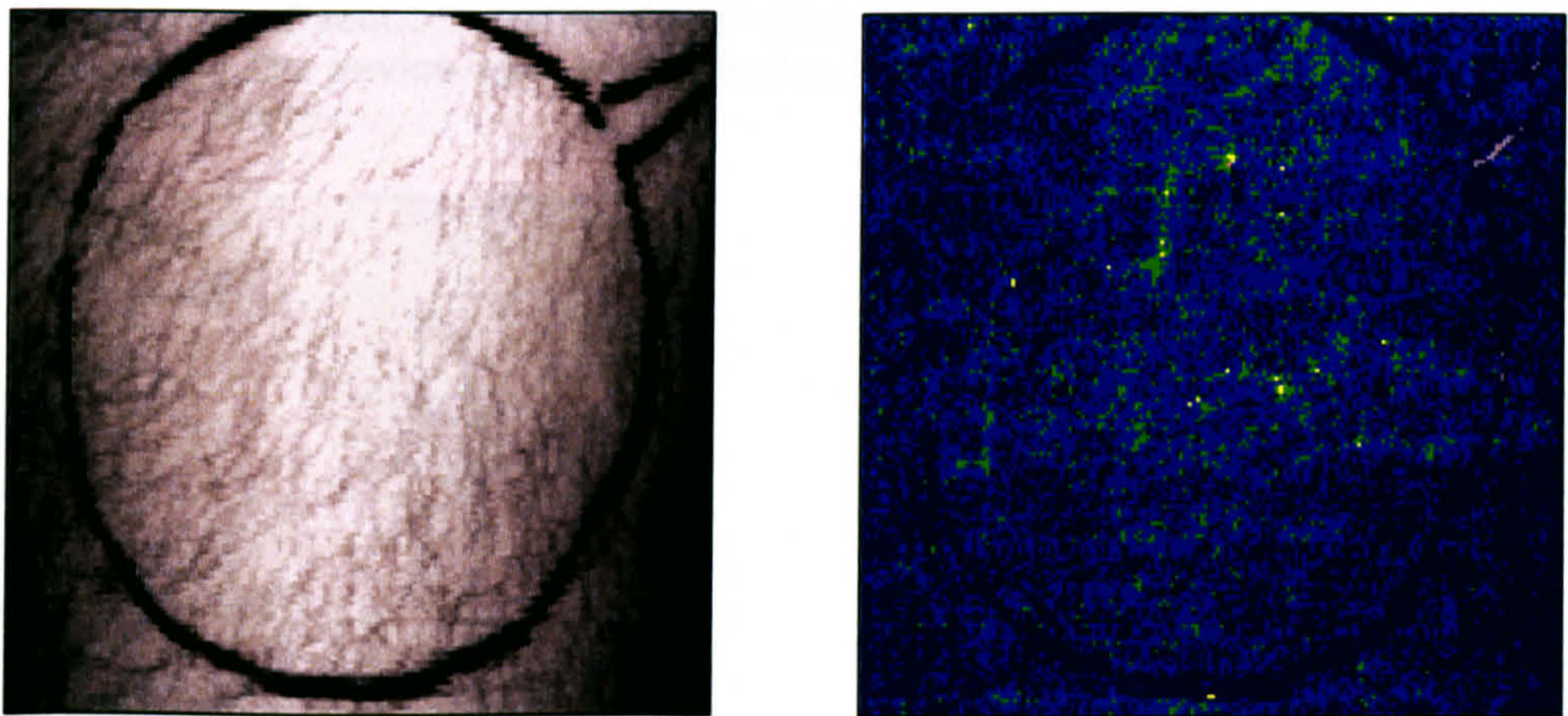


Figure 20 The same DC and flux image as shown above after 1 hour of TNP therapy at subatmospheric pressure of 125mmHg (scan distance: 37cm)

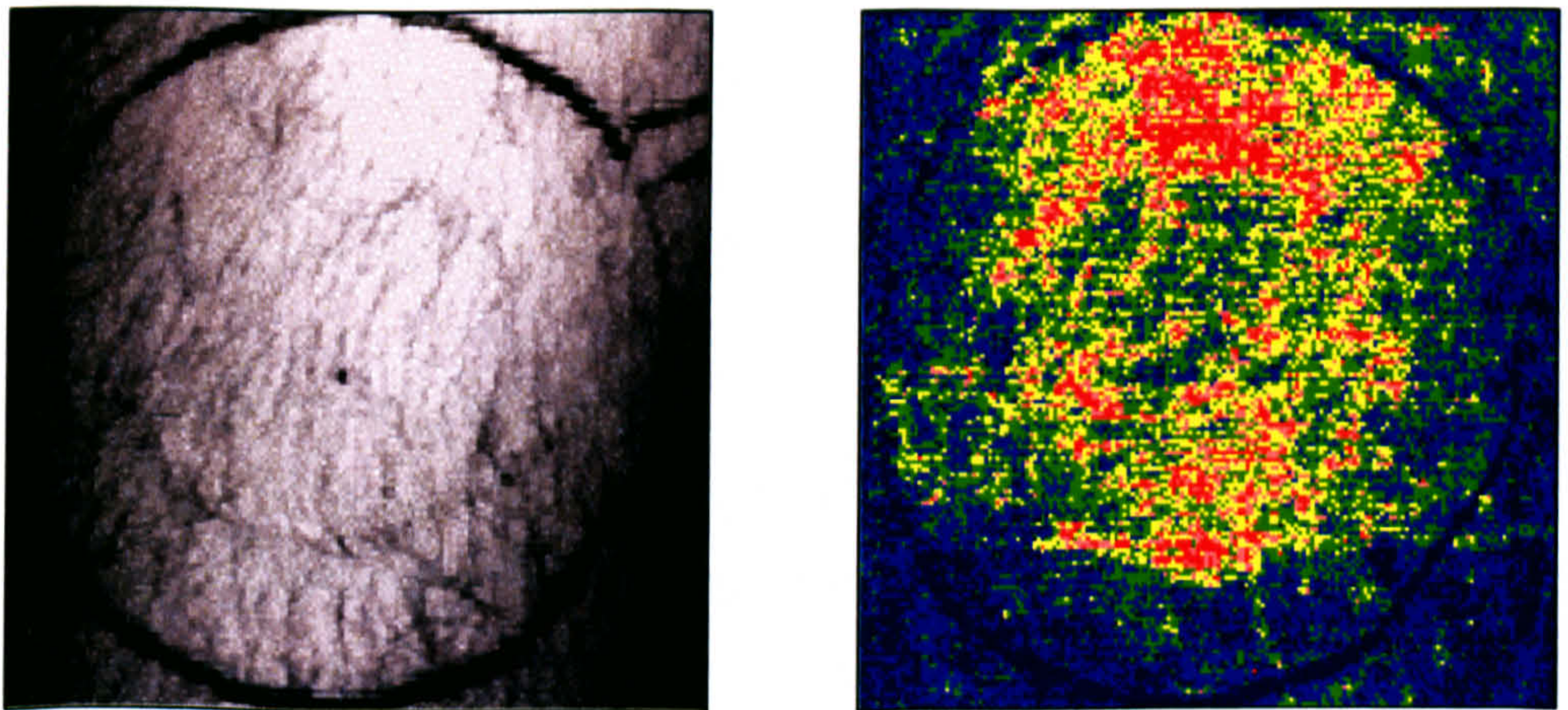


Figure 21 DC and flux image of right calf skin using the small standard TNP device, blue ring and Spenco™ pad for 5 hours at subatmospheric pressure of 125mmHg. The device is identical to that shown in Figure 6 (scan distance: 40cm)

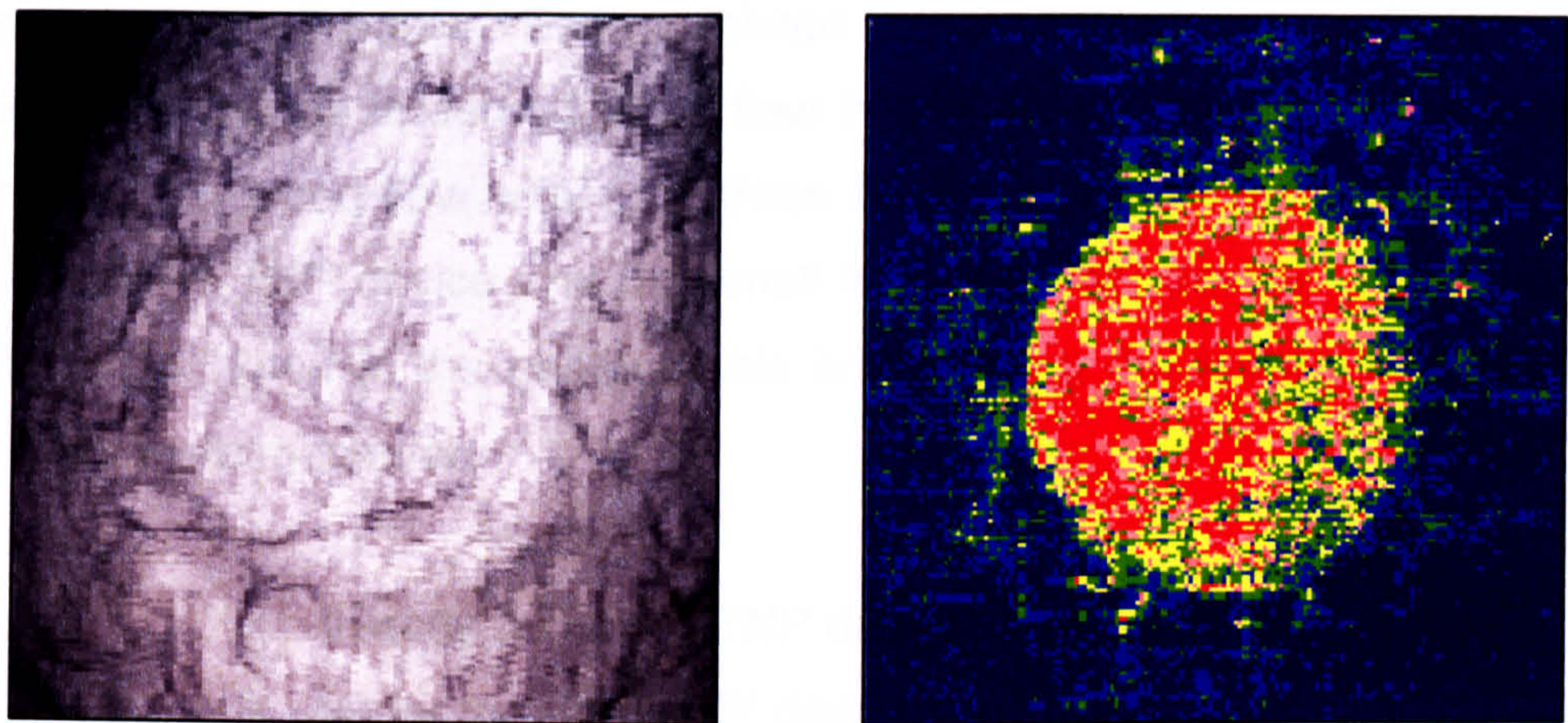
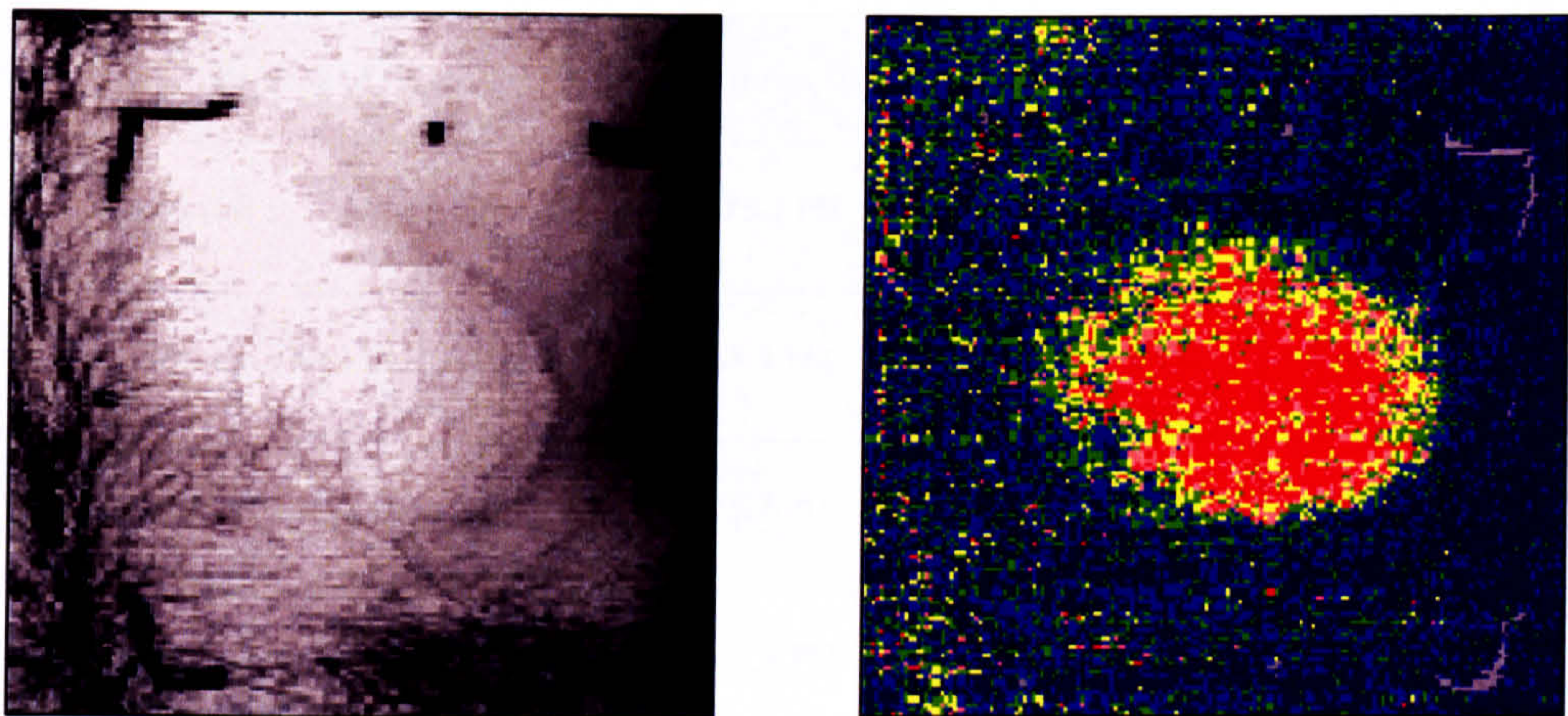


Figure 22 DC and flux image of left sided chest skin using the small TNP irrigation device, blue ring and Spenco™ pad for 30 minutes at subatmospheric pressure of 125mmHg. The device used is identical to that shown in Figure 10 (scan distance: 30cm)



Statistical analysis, using the LDPI software on the results of Figure 21 and Figure 22, is shown in Table 7 below. Each pixelated colour represents a perfusion unit (PU), which corresponds to dermal blood flow in that area. The PU values in the table represent the blood flow within the three surfaces under investigation using the small standard TNP device and the small irrigation TNP device. By drawing around the areas noted in the photographic image using the software provided, perfusion units were measured:

- i. directly under the TNP device
- ii. under the Spenco™ dermal pad alone
- iii. beyond the pad on surrounding normal skin

Table 7 Perfusion units (PU) measured directly under TNP therapy. A small standard TNP device and a small irrigation TNP device were tested. Measurements of flux were made directly under, surrounding and beyond the device.

Surface of skin	mean	SD	Min	Max
Skin under small <u>standard</u> TNP device	415.1 PU	152.7 PU	34 PU	819 PU
Surrounding skin under the Spenco™ pad	88.3 PU	51.3 PU	0 PU	640 PU
Normal skin beyond the Spenco™ pad	75.7 PU	37.6 PU	0 PU	640 PU
Skin under small <u>irrigation</u> TNP device	653.9 PU	280.5 PU	111 PU	2273 PU
Surrounding skin under the Spenco™ pad	208.1 PU	96.7 PU	44 PU	1230 PU
Normal skin beyond the Spenco™ pad	204.8 PU	93.8 PU	41 PU	2015 PU

The results demonstrate that a significantly raised mean flux value in the area under TNP therapy is apparent ($P<0.0001$, t-test) compared to the mean flux values of normal skin in both the standard and irrigation TNP devices (5.5-fold and 3.2-fold, respectively). These results are consistent with those of Morykwas et al. (1997) who observed in full thickness experimental swine skin wounds (n=5) a 4-

fold increase in perfusion units between TNP treated and baseline readings. There is no significant difference between the flux values under the Spenco™ pad compared to the values representing normal skin (83.3PU and 75.7PU for the standard device; 208.1PU and 204.8PU for the irrigation device, respectively). Comparisons between the three different skin sites can be made with confidence because the LDPI can scan the whole site in one sitting. Caution is required when attempting to compare the efficacy of the two TNP devices directly in terms of perfusion/flux. The observed differences in flux values between the standard and irrigation devices (415.1PU and 653.9PU, respectively) are most likely due to differences in the skin surface quality, texture and thickness under investigation (normal right calf and normal left chest skin), the duration of application (5 hours versus 30 minutes) and technical differences with the machine.

Given that the pressures within each device are the same, the observed difference in flux between the devices is less likely to be due to the differences in their design but most likely due to the differences in skin quality and suction time. Repeat studies need to be performed using both these devices to confirm this further.

3.3.3.3 Bonney's Blue Dye Testing

The first tests using the hydrocolloid base were performed on the laboratory bench using a large irrigation TNP device as shown in Figure 15 (on page 94) before similar tests were performed on normal skin as shown in Figure 16.

Figure 17 shows how the blue dye distributes itself uniformly over the skin and is contained within the ring design. Nonetheless, two significant problems of using the hydrocolloid base as protection to the skin are well illustrated. Firstly, the hydrocolloid material is easily deformed after a few hours of suction. This results in a loss of protection as noted in the photograph by the evidence of the blue rim of the ring design. Secondly, due to the failure of the hydrocolloid base in protecting the normal skin, there is a significant imprint on the skin surface.

The pressure effects noted using this design might compromise injured and ischaemic skin such as donor site wounds or burns. Also, there was concern about whether a circumferential imprint around the TNP device would impede dermal blood flow in a lateral direction at the level of the rim.

Further work using this hydrocolloid design ceased after it became clear that the hydrocolloid product deformed quickly as it took up moisture and could not withstand subatmospheric pressures of 125mmHg for up to 48 hours without losing its protective barrier. Moreover, the adhesive properties of the product added to the discomfort noted when the device was removed from both normal skin and injured skin. Removal of the device to inspect or record parameters at the skin surface broke the seal and damaged the hydrocolloid base.

The above dye tests were repeated using the Spenco™ dermal pad protective base design. The results of these tests are shown in Figure 23 through to Figure 25 on page 104. The dye is uniformly distributed on the skin surface and no leakage is noted beyond the ring. In these tests, the Spenco™ product remains intact; the skin is protected from the blue rim by the dermal pad and can be easily removed for inspection of the skin surface as necessary.

3.3.3.4 Radiopaque Dye Testing

These results supplemented the Bonney's blue dye tests, confirming the uniform distribution of the dye throughout the device on repeated testing. The radiopaque contrast could be seen permeating through the lower foam chamber and onto the 'skin' surface. The contrast could be seen within the upper foam chamber and leaving through the exit tubing after connection to the pump. 2mls of air insufflation following 2mls of contrast helped expel irrigant fluid from the tubing. Despite repeated washouts with Normal Saline, small traces of contrast could still be seen throughout the device, mainly in the upper foam chamber.

Figure 23 Small irrigation TNP device under suction using the Spenco™ dermal pad. Note the black ink used to mark out the boundary.

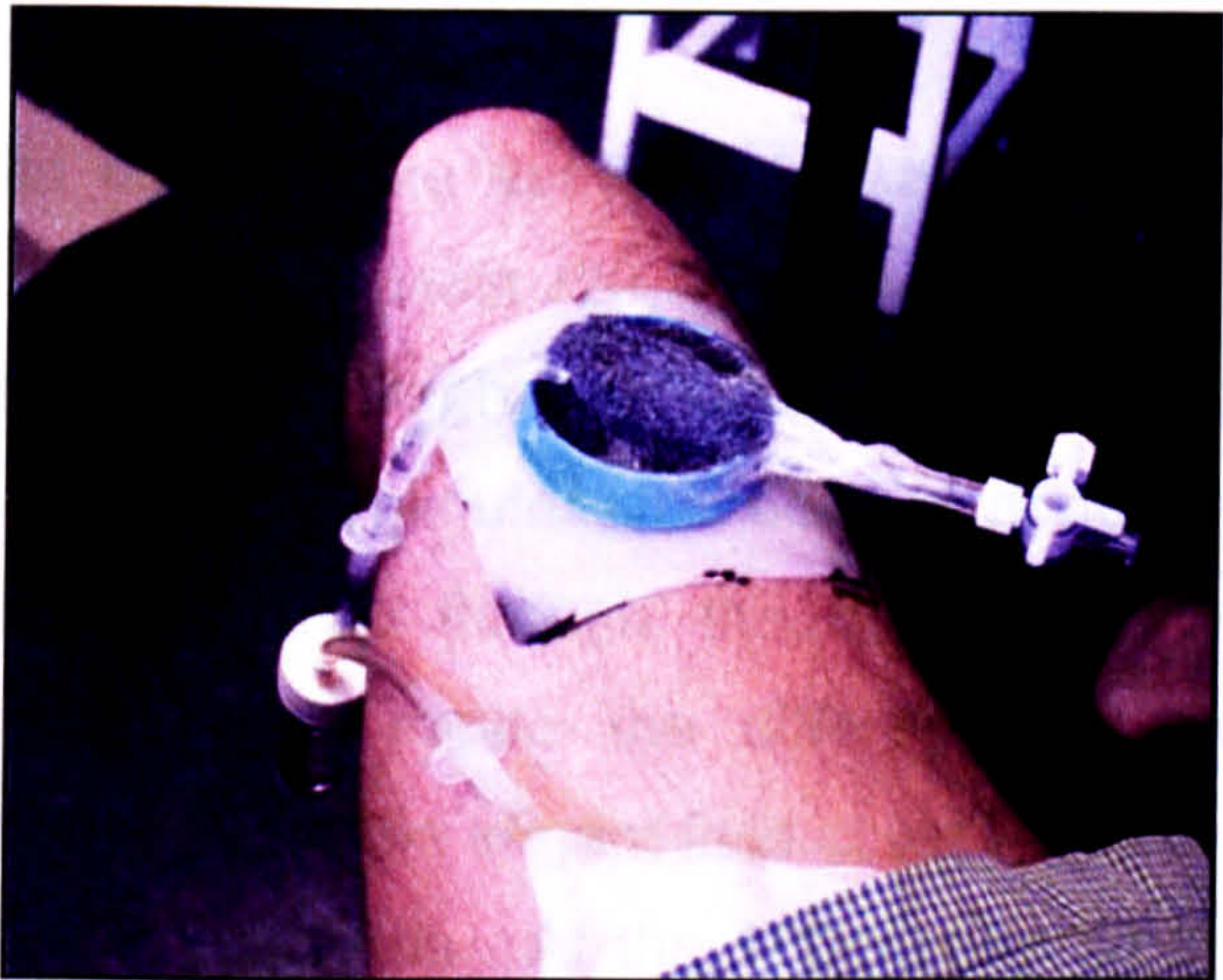
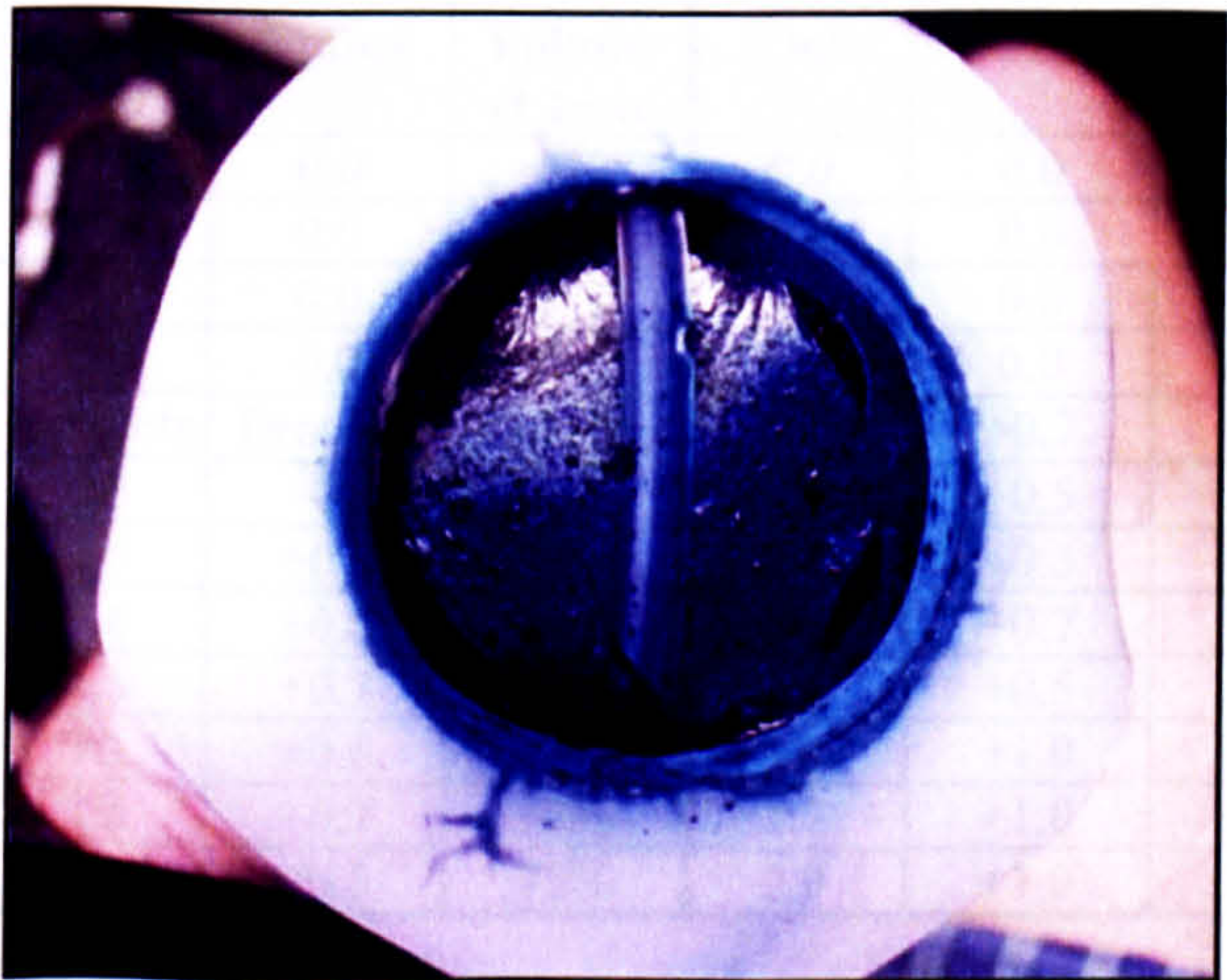


Figure 24 Uniformity of dye without evidence of leakage on removing the device from the skin. Note that the blue rim is still well protected by the dermal pad.



Figure 25 View of underside of small irrigation TNP device. Note the perforations within the Tegaderm™ dressing (after removing the lower foam chamber).



3.3.3.5 Fluid Volume Measurements on Normal Skin

The results demonstrate some variability between the onset of fluid that was collected in the three tests (20, 40 and 50 minutes). The differences noted in ‘priming’ each system might be explained by the variability of each individual TNP device used. By 100 minutes, the devices appeared to reach equilibrium, in that irrigant fluid delivered through the system provided near constant volumes retrieved from it. Despite the differences between the tests, at 2 hours approximately 75% of the irrigant fluid could be collected through each TNP device over the first 60 seconds. At the 120-minute time point, 2mls of Bonney’s Blue was instilled through the device instead of Normal Saline. Blue dye was seen immediately following the onset of suction at 1 minute, confirming that the same fluid instilled was likely to be retrieved during each collection.

It was possible that some fluid remained within the device, even during prolonged (10 minute) suction, although the volume would remain low as suggested by the results in Table 8. The volume of fluid potentially trapped within the system was not measured.

Table 8 Results of 3 similar tests involving 10-minute irrigations on normal skin using the small TNP irrigation device. 2mls of Saline is instilled every ten minutes for 2 hours. Fluid volumes collected at 1 minute and at a further 9 minutes are recorded

Test No.	1			2			3		
Time (in minutes)	1 min	9 mins	% Volume at 1min	1 min	9 mins	% Volume at 1min	1 min	9 mins	% Volume at 1min
0	0.0	0.0	0%	0.0	0.0	0%	0.0	0.0	0%
10	0.0	0.0	0%	0.0	0.0	0%	0.0	0.0	0%
20	0.0	0.0	0%	0.8	0.0	40%	0.0	0.0	0%
30	0.0	0.0	0%	1.2	0.0	60%	0.0	0.0	0%
40	Droplets	Droplets	Droplets	1.0	+0.7	50%	0.0	0.0	0%
50	2.3	+0.5	115%	1.0	+0.5	50%	0.0	+0.5	0%
60	1.5	+0.6	75%	1.7	+0.5	85%	0.1	+3.0	5%
70	1.6	+0.5	80%	1.5	+0.7	75%	0.0	+0.0	0%
80	1.5	+0.7	75%	2.0	+0.5	100%	0.7	+0.4	35%
90	1.5	+0.6	75%	1.5	+1.0	75%	2.0	+0.4	100%
100	1.5	+0.7	75%	1.2	+1.0	60%	1.5	+0.1	75%
120	1.5	+0.5	75%	2.0	+1.0	100%	1.5	+0.1	75%

3.3.4 Evaluating the small irrigation device on acute dermal wounds

The surfaces of the TNP treated wounds are pinker when compared to the surrounding wound surface. No active bleeding was noted following TNP therapy on either the donor site or burn wounds. It was noticeable on the acute donor site wound that the effects of suction therapy on the wound surface might extend up to 1cm beyond the area of suction, since a circle of clotted blood was noted 1cm beyond the foam (see Figure 26). This suggests that subatmospheric pressure is having an effect on the wound surface beyond that of the foam, possibly dragging surrounding wound fluid into the TNP system.

There was no evidence that the effects of TNP therapy using the TNP devices caused any harm to acute donor site wounds and partial thickness burn wounds. Using the Spenco™ dermal pad, no pressure effects from the base of the ring were seen after TNP therapy.

No direct wound healing complications were noted in any patient studied. One patient with a donor site wound (not involved in the wound fluid study described in the following chapters) developed a superficial *Staphylococcus Aureus* wound infection four days following TNP therapy using the device. The device became dislodged during therapy, which might have exposed the wound surface to an increased risk of contamination. No TNP-treated burn wound became infected.

All the patients who agreed to participate in the following studies in this thesis completed the 48-hour TNP therapy using these devices, except in one case in which accidental damage to the device occurred. The level of discomfort was variable between patients, yet patient compliance remained 100%. During TNP device testing on wounds, a number of young patients had also been studied without difficulty (see Figure 28).

Figure 26 Examples of selective TNP therapy to donor site wound surfaces. Note the pale area around the foam, which suggests a wider area of TNP influence.

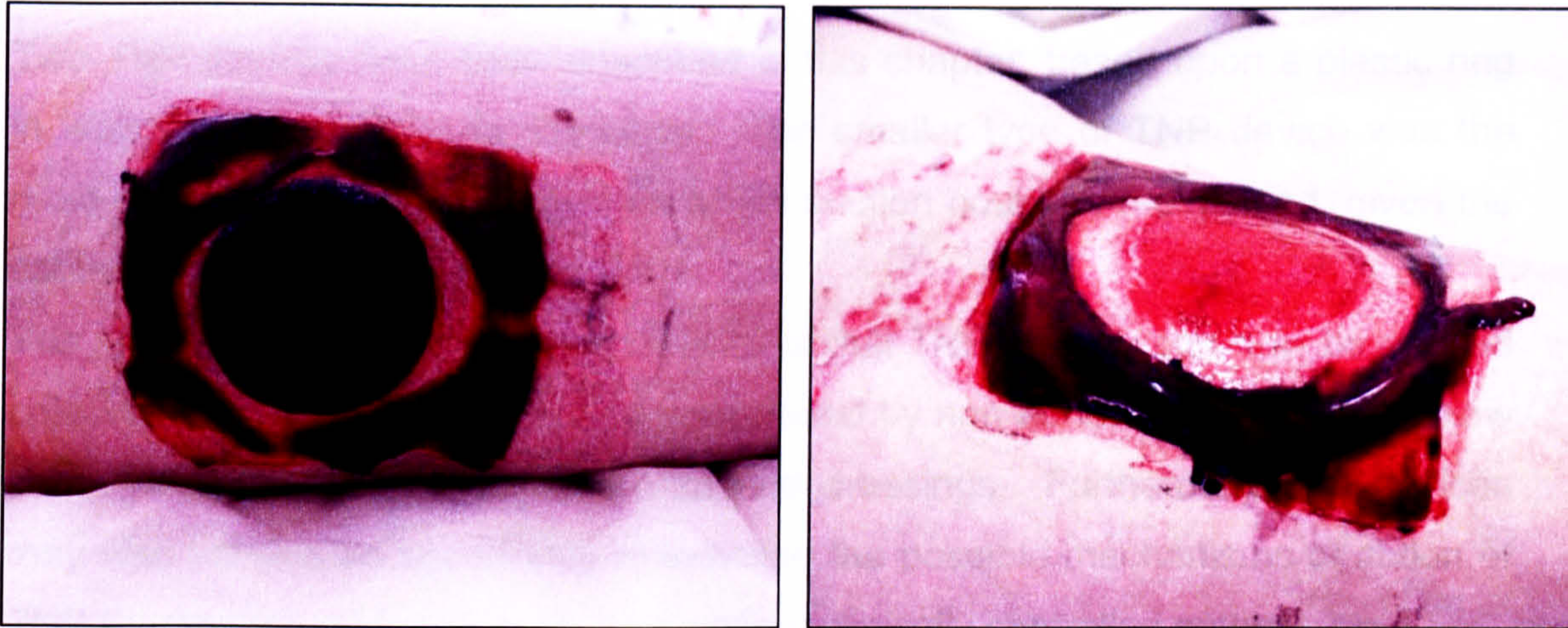


Figure 27 The use of a TNP device on a superficial burn wound. Note the position of two sets of biopsy samples in this case



Figure 28 A contented young boy undergoing standard TNP therapy to a burn on his left leg. A device has been positioned distally as a control



3.4 Discussion

Two TNP devices have been described in this chapter, based upon a plastic ring in which V.A.C.™ foam is contained. The smaller type of TNP device was the most effective on the extremities on which suction could be maintained, given the convexity of the skin surface.

The principle advantage of these devices is that TNP therapy can now be applied selectively over open wounds, and surrounded by non-adherent surfaces, to allow comparisons to be made against standard dressings. Furthermore, the devices may also provide an opportunity to examine the possible mechanisms of action of TNP therapy more closely in an intra-patient clinically controlled setting.

The second part of this study was concerned with testing and validating the TNP devices to ensure their reliability and safety on unwounded and wounded skin. The topical negative pressures measured between the two types of TNP device and within the chambers of the irrigation device, support the view that suction pressures are distributed equally across the foam(s) within all the TNP devices described.

Using the LDPI to map blood flow characteristics in skin, these tests also confirm that the open-cell nature of the PVA foam provides a uniform distribution of topical negative pressure directly under the foam area, within both the standard and irrigation TNP devices with an appropriate skin protector. Indeed, when the base of the outer plastic ring is padded using the Spenco™ dermal pad, for example, the 'halo' effect is prevented as demonstrated with the LDPI tests. The flux distribution also suggests that the TNP devices *do* limit the effects of suction therapy to the area of contact when measured using laser Doppler techniques. Recently, Droog et al. have highlighted some of the difficulties in LDPI interpretation (Droog et al., 2001). They demonstrated that significant variations in perfusion values might occur due to the curvature of the skin under LDPI and differences in scanning distance. In this case, the area undergoing scanning was no more than 10x10cm with minimal skin curvature. The scanning distances remained between 30 and 40 cm only, with no difference in Figure 19 and Figure 20. This allows for direct visual comparisons between images to be made more accurately. Ambient light intensity, temperature and humidity were standardised as much as possible in all these tests.

The dye tests confirm that the irrigant fluid permeates without hindrance through the lower foam chamber and onto the skin surface. Air insufflation ensures that no fluid is left in the irrigant tubing. The passage of dye, in both the Bonney's Blue and Urograffin[®] experiments, through the two chambers of foam is unimpeded and the four 1.5cm perforations appeared adequate to allow the suction of fluid between the chambers. The distribution of blue dye is contained within the foam and no leakage beyond the ring is apparent using the irrigation TNP device. In these tests, the Spenco[™] dermal pad appears more superior and the skin remained protected from pressure effects of the rim of the plastic ring that might have been a potential problem using this device. Furthermore, this protective barrier does not lose its competence when the device is removed for inspection of the surface, for example, which is not the case using the hydrocolloid barrier.

Early variability of fluid collection exits using the irrigation TNP devices on normal, dry skin, both within and between devices. To ensure that wound fluid/irrigant fluid could be reliably retrieved from the system at frequent time points, 2mls was chosen as the optimum volume to use when tested on dry skin. After 2 hours, it appeared that approximately 75% of the fluid instilled is retrieved over the first 60 seconds.

The safe use of the TNP device to deliver TNP therapy on acute dermal wounds is demonstrated in studies performed over an 18-month period. There was no subjective evidence of a delay to wound healing or damage to the surface of the wound during TNP therapy for up to 2 days using this new system. Following removal of the TNP sponge after 48 hours of therapy no bleeding was seen, suggesting that the surface of the wound was not traumatised clinically. In agreement with other studies using TNP therapy on acute human donor site wounds, TNP did not cause significant discomfort (Genecov et al., 1998). Indeed, throughout prolonged TNP therapy on both wound types, patients slept with the device switched on. No conclusions can be made with respect to the efficacy of TNP therapy on acute donor site or partial thickness burn wounds at this time. Patients are still being followed up.

3.5 Conclusion

This study chapter has detailed the design of two TNP devices, based on commercially available V.A.C.[™] dressings, which can be used on acute open dermal wound surfaces. The 'standard' TNP device mimics the use of the TNP dressings currently available commercially. The 'irrigation' TNP device provides a further opportunity to allow collection of wound fluid frequently from the same site under investigation.

The validation tests help to confirm that the devices are intended to do what they were designed to do; which is to provide discrete TNP therapy to a wound surface and also to allow frequent and specific wound fluid sampling over the surface of the wound under investigation.

The use of the 'irrigation' TNP device is used exclusively in the following chapters.

4 Temporal analysis of Total Protein in wound fluid under the influence of TNP following partial thickness dermal injury

4.1 Introduction

Modulating the extracellular wound fluid environment has been suggested as one mechanism by which Topical Negative Pressure (TNP) Therapy may influence wound healing. The design of an irrigation TNP device described in Chapter 3 provides an opportunity to test this hypothesis.

In order to determine whether TNP therapy can modulate the extracellular wound fluid environment, the aim of this study was to retrieve fluid from the wound surface and measure the total protein content during cyclical 'on/off' TNP therapy over a 48-hour period using the newly developed irrigation TNP device. Two human dermal wounds were chosen; the donor site wound and the partial thickness burn wound. TNP therapy has been used safely on both of these wound surfaces in a clinical and experimental setting as described in Chapter 3.

4.2 Materials and Methods

4.2.1 Study Design

A prospective, longitudinal study was devised based on patients attending the Department of Plastic Surgery and Burns Unit at the Stoke Mandeville NHS Trust. Using the irrigation TNP device, intermittent TNP therapy was applied to acute donor site wounds at the time of surgery, or to partial thickness burns on admission to the Burns Unit, for a period of up to 48 hours. TNP therapy was applied uniformly, every six hours over the duration of the study period, and wound fluid samples collected during both the on and off 6-hourly cycles.

4.2.2 Patient Selection and Details

Ethical approval was obtained from the Aylesbury Vale Local Research Ethics Committee to study the effects of TNP therapy on human donor site wound healing (Project Code: NC903) and human burn wound healing (Project Code: NC973). Approval was also granted to allow for fluid collection and obtaining skin biopsies (see Chapter 5 on page 135). In addition, the history and examination of patients with burns were recorded and digital photographs taken of TNP treatment sites.

Patients were selected in accordance to the following criteria:

Inclusion criteria:

- a. Age group between 12 – 90 years.
- b. Any donor site wound or any partial thickness burn on the trunk or limbs in adults and children that may or may not require operative treatment.
- c. Fully conscious adult patient who is able to give informed consent.
- d. Fully conscious child patient, aged 12 years or older, who is either able to understand the treatment proposed and who are willing to

give informed consent, or a willing child whose parents/guardian wish to provide consent on the child's behalf.

e. Total percentage body surface area burned between 1-40%.

Exclusion criteria:

- a. Burns to face and perineum.
- b. Presence of inhalation injury.
- c. Presence of any other serious medical condition or generalised ill health.
- d. Inability to give informed consent.

Small irrigation TNP devices, previously constructed under sterile conditions, were applied immediately on to donor site wounds created using an air-driven dermatome (Zimmer Ltd., UK) in the operating theatre (see Figure 29 on page 116). Small irrigation TNP devices were applied to clinically homogeneous partial thickness burns (see Figure 30 on page 116). The starting time point for each burn wound fluid collection experiment depended on the arrival of each burn patient to the Burns Unit at Stoke Mandeville Hospital. Subatmospheric pressures of 125mmHg were used throughout all of the experiments. The following patients were enrolled into the study²:

Donor site wounds: (n=7)

Six patients consented for TNP therapy of their donor site wounds using the irrigation TNP device. One patient consented for two donor site wound fluid collections. Their details are summarised in Table 9 on page 114.

Partial thickness burn wounds: (n=5)

Three patients consented for TNP therapy of their partial thickness burn wounds using the irrigation TNP device. Two of these patients consented for two separate areas for TNP therapy and burn wound fluid collection. Their details are summarised in Table 10 on page 115.

² For clarity in this thesis, the same identification (ID) numbers are assigned to the same patients throughout this study and subsequent study chapters in which fluid or tissue is obtained

Table 9 Summary of Patients with donor site wounds enrolled in chapter 4

Donor Site ID	Age	Sex	Wound site	Dermatome depth	Start time	Stop time	History	Past Medical History	Current Medication
1	74	F	Right thigh	0.008 inch	In theatre	45 hours	SSG to excised scalp tumour	Hypertension	Bendrofluazide
2	42	M	Right thigh	0.01 inch	In theatre	48 hours	SSG 19 days post 4% FTB	Epilepsy, Psoriasis	Phenytoin
3	87	M	Right thigh	0.008 inch	In theatre	30 hours*	SSG to excised scalp tumour	Cerebral Ischaemia Glaucoma	Aspirin (stopped) Timolol
4	19	F	Right thigh	0.008 inch	In theatre	48 hours	SSG 11 days post 13% PTB	N/a	N/a
5	32	F	Right thigh	0.01 inch	In theatre	48 hours	SSG to excised left leg tumour	Psoriasis	Contraceptive pill
6a	56	M	Abdomen	0.008 inch	In theatre	48 hours	SSG to 7 days post 40% PTB & FTB	N/a	N/a
6b	56	M	Abdomen	0.008 inch	In theatre	48 hours	SSG to 7 days post 40% PTB & FTB	N/a	N/a

SSG – split skin graft
FTB – full thickness burn
PTB – partial thickness burn
* Fluid collection stopped at 30 hours after device became dislodged from experimental site

Table 10 Summary of patients with burn wounds enrolled in chapter 4

Burn ID	Age	Sex	Burn wound site	Burn type	Clinical depth of burn	% TBSA	Start time	Stop time	Past Medical History	Current Medication
A	44	M	Right chest	Hot fat	Deep partial thickness	4.5%	12 hours	46 hours	N/a	N/a
Ba	19	F	Right buttock	Scald	Deep partial thickness	9% DPT 4% FTB	10 hours	30 hours	N/a	N/a
Bb	19	F	Left buttock	Scald	Deep partial thickness	9% DPT 4% FTB	10 hours	30 hours	N/a	N/a
Ca	26	M	Left leg	Flame	Superficial partial thickness	17% SPT	16 hours	49 hours	Asthma	Ventolin
Cb	26	M	Posterior leg	Flame	Superficial partial thickness	17% SPT	16 hours	49 hours	Asthma	Ventolin

TBSA – total body surface area
SPT – superficial partial thickness burn
DPT – deep partial thickness burn
FTB – full thickness burn

Note the delay in start time of TNP therapy

Figure 29 Application of small TNP device to a donor site wound. The irrigation port and evacuation tube are above and below respectively (Donor site ID 3)

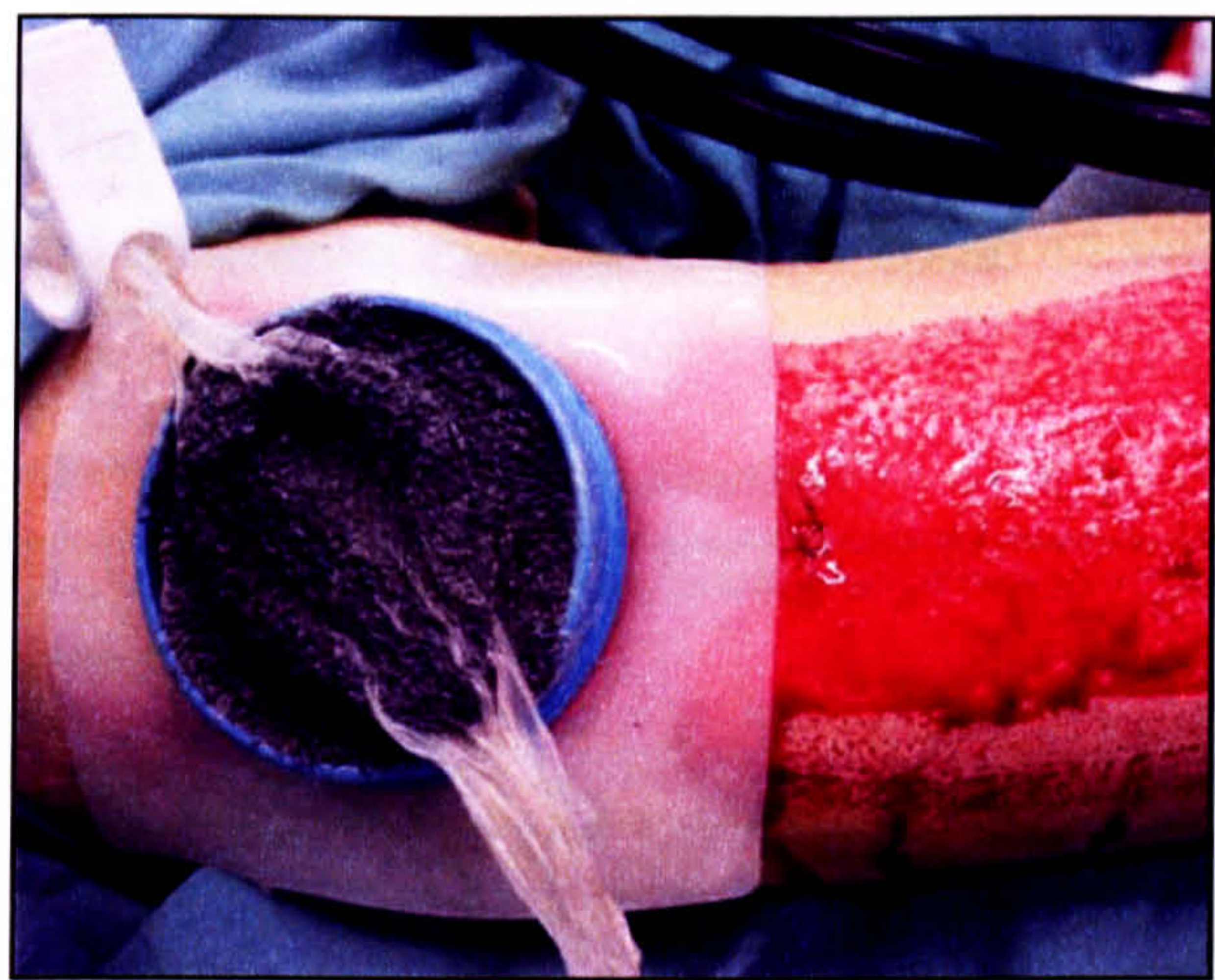


Figure 30 Application of small TNP device to one area of deep partial thickness burn wound (Burn ID A)

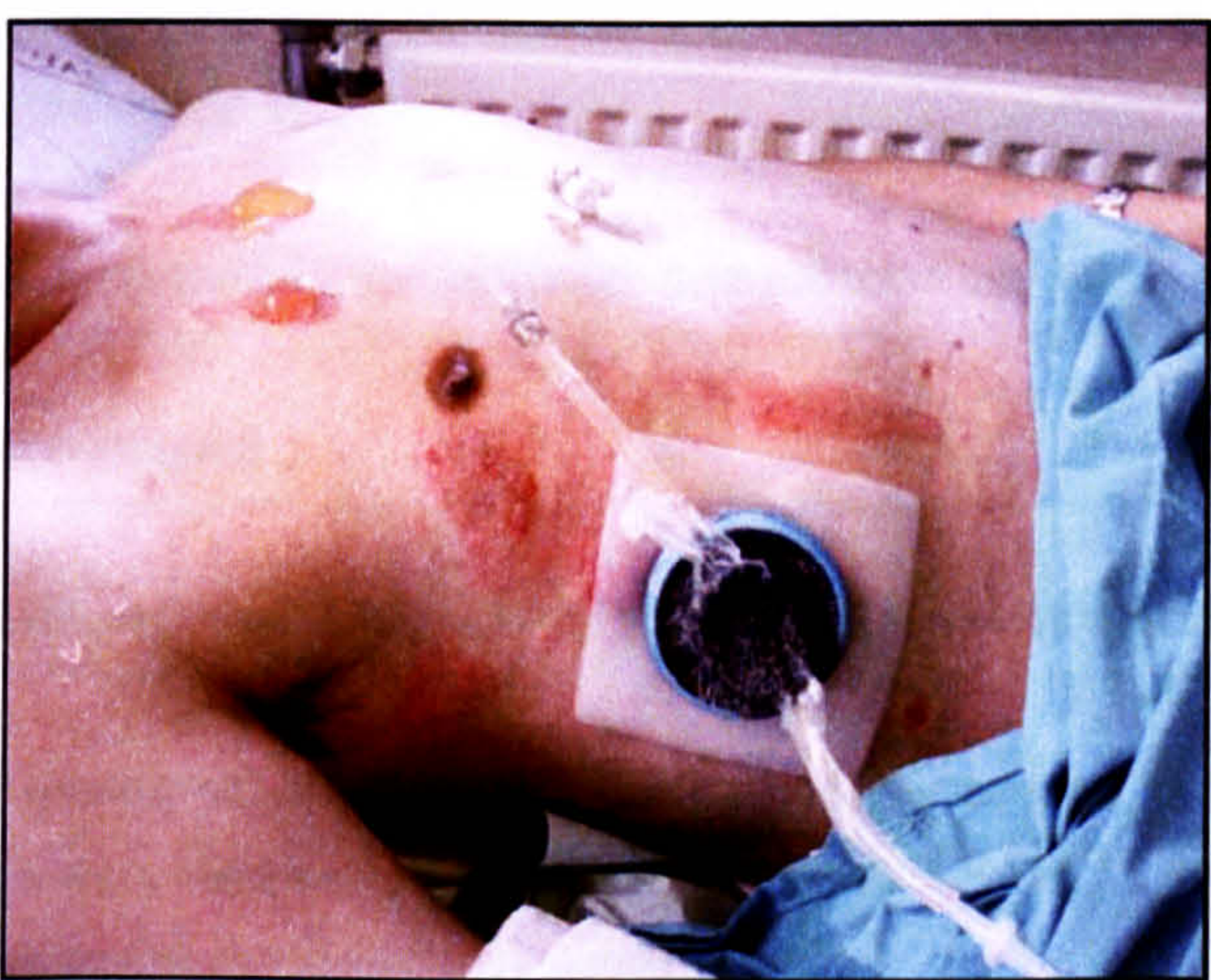


Figure 31 Donor site wound under suction with sterile container in situ for wound fluid collection



4.2.3 Wound fluid collection and storage

At each of the wound fluid collection times, an identical procedure was performed for each fluid collection, as described in Table 11 below.

Table 11 Method of wound fluid collection

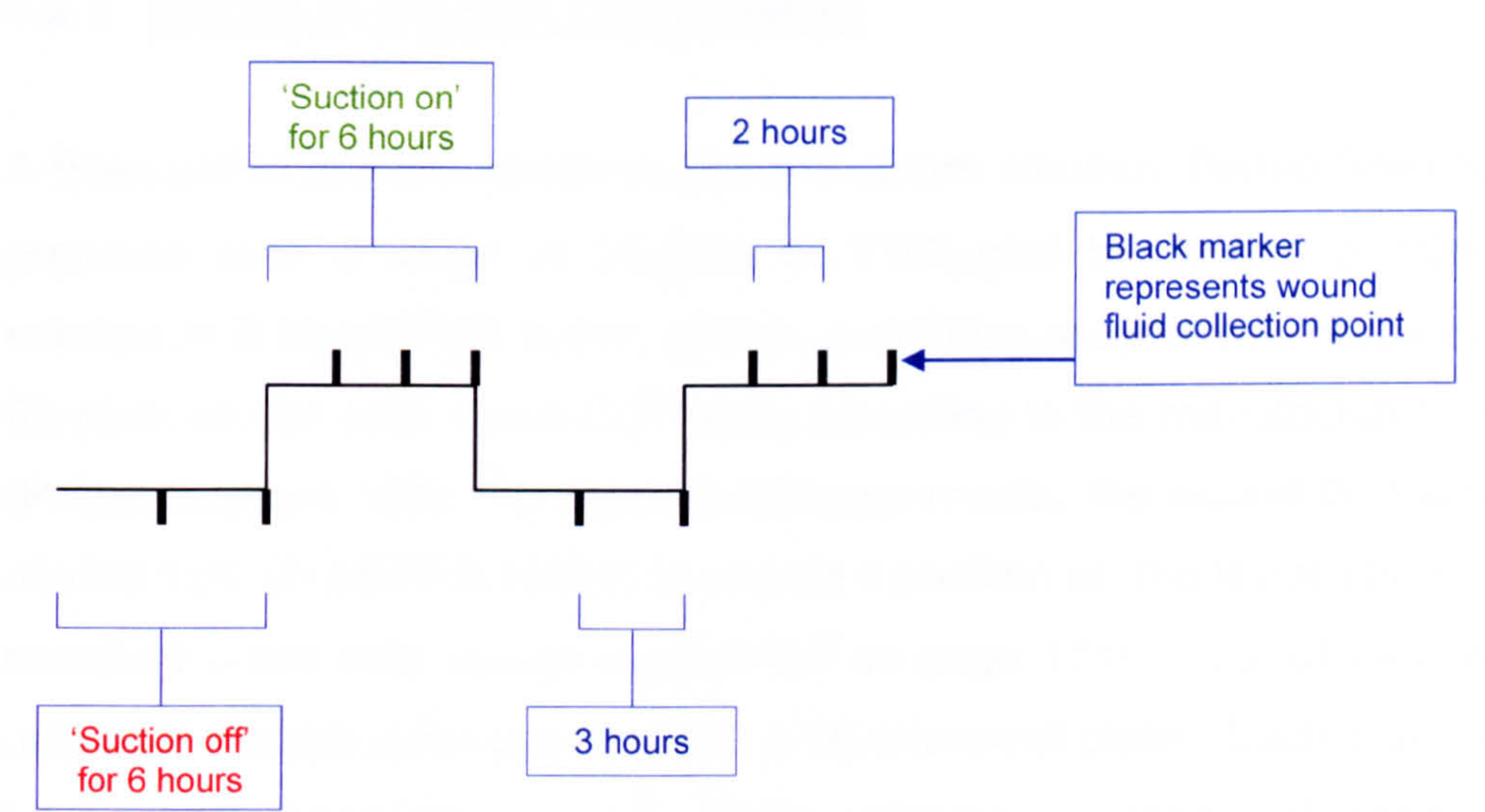
1.	TNP therapy stopped (if during the 'suction on' phase) and the foam dressing allowed to rise to its pre-suction position
2.	A sterile fluid collection container was connected close to the exit port of the device (see Figure 31)
3.	2mls of Normal Saline (irrigant fluid) gently instilled through the lower irrigation port
4.	2mls of air instilled through the irrigation port
5.	One minute was recorded to allow irrigant fluid to distribute throughout lower chamber of device and onto the wound surface
6.	Suction started and wound/irrigant fluid collected into sterile container over a further one minute
7.	Suction stopped
8.	The volume of wound/irrigant fluid in the container was measured
9.	TNP therapy resumed (if during the 'suction on' phase) or left off (if during the 'suction off' phase)
10.	Fluids were transferred to 1.5ml vials and centrifuged for 5 minutes at 4000g (Eppendorf 5416, Netheler, Germany) to remove cells/debris
11.	Aliquots of the supernatant (400µl) were snap frozen in liquid nitrogen
12.	Samples were stored at -70°C prior to analysis

The time from wound fluid collection to snap freezing was less than 10 minutes in each case. The donor site wound fluid from Patient ID 1 (the first patient using this device) was snap frozen immediately prior to storage. In this case, the wound fluid was not centrifuged until after thawing. See Results section 4.3.

Three sets of wound fluid were collected every 2 hours during each of the 6-hourly 'suction on' phases and two sets of wound fluid collected every three hours during each of the 'suction off' phases up to two days from the onset of injury (see Figure

32 below). The actual time, the experimental time and the volume of wound fluid at each collection point were recorded. Reprints of the data collection sheets are given in Appendix 10.1 and 10.2.

Figure 32 Schematic diagram to demonstrate wound fluid collection time points



4.2.4 Total Protein Assay of fluid samples

Principles:

The labelled wound fluid samples (n=194) were analysed using a bicinchoninic acid (BCA) protein assay technique (Perbio Science, UK). This method combines the reduction of copper ions by proteins (the biuret reaction) with the highly sensitive and selective colourimetric detection of Cu⁺ cations in the samples when combined to bicinchoninic acid as a complex. A purple-coloured reaction product is formed by the chelation of two molecules of BCA with one cuprous ion; the intensity of colour is proportional to the protein concentration.

The BCA-Protein Reaction:

1. Protein (peptide bonds) + Cu²⁺ $\xrightarrow{\text{OH}^-}$ tetradentate-Cu⁺ complex
2. Cu⁺ + BCA(x2) \longrightarrow BCA + Cu⁺ complex (purple coloured)

This water-soluble complex exhibits strong absorbance at 562nm that is linear with increasing protein concentrations over a broad working range of 20µg/ml to 2000µg/ml.

4.2.5 Method for the BCA Protein assay:

A fresh set of protein standards (bovine serum albumin, Perbio Science, UK) was prepared over a range of 25µg/ml to 1750µg/ml by diluting a 2.0mg/ml stock solution in 0.1M HEPES buffer, pH7.4. A working reagent was prepared by mixing 50 parts of OH⁻ with 1-part Cu²⁺ ions, according to the manufacturers instructions (Perbio Science, UK). To achieve optimum results, the wound fluid samples were diluted 1:50 (in HEPES buffer) to ensure a position on the linear part of the protein standard curve (see results section 4.3 on page 121). 25µl of each standard or unknown sample were pipetted into a 96-microwell plate. Each plate was able to house one complete set of patient samples, a range of protein standard concentrations and blanks (containing 25µl of HEPES buffer alone) in triplicate. 200µl of working reagent was added to each well, and the plate was covered and incubated at 37°C for 30 minutes.

After cooling the plate for 5 minutes to room temperature, the colour change was measured photometrically at 550nm on a plate reader (Titertek Multiskan MCC/340, Labsystems, Finland). The average reading from the three blanks was subtracted from the triplicate readings for each protein standard and unknown sample.

A standard curve was prepared by plotting the average blank-corrected reading for each protein standard versus its concentration in µg/ml. Using the standard curve, the protein concentration for each of the unknown samples was determined by formulating a linear equation and determining protein concentrations from the absorbance readings.

Automated Total Protein Measurements

Total Protein was also measured photometrically using an automated Biuret method on a COBAS MIRA Plus (Roche, UK) courtesy of Dr. Joanna Sheldon at the Protein Reference and Immunopathology Unit, St. George's Hospital, London. A Total Protein reagent (ABX Diagnostics, Montpellier, France) was used which is standardised against bovine albumin.

Quality Control: coefficient of variation 1.4% for low, medium and high concentrations.

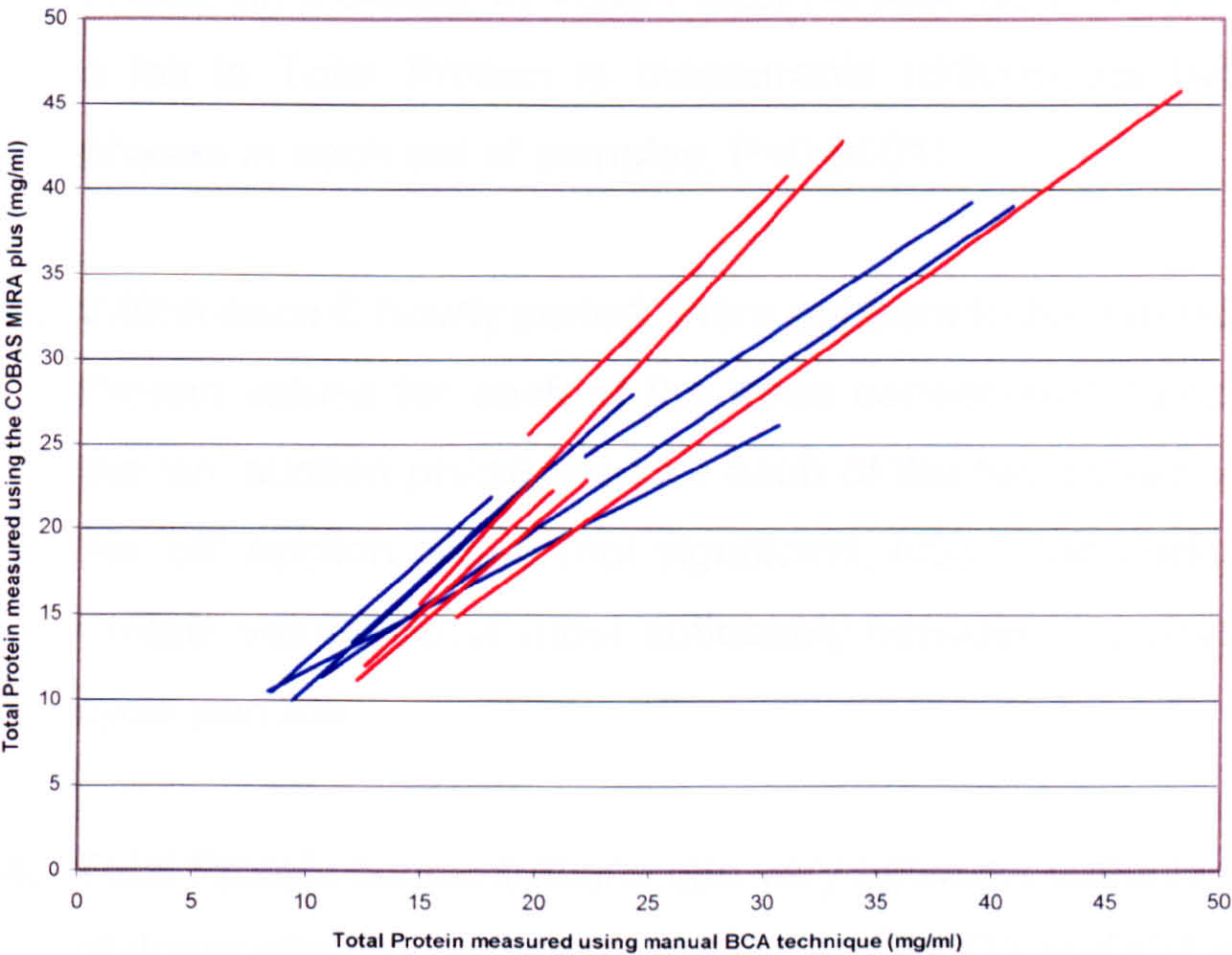
Statistical analysis (ANOVA) was performed using STATVIEW 5.0 (SAS Institute, Ltd.) and DATADESK 6.0 (Data Description, Inc.) software, with the assistance of Dr. Brian Shine, University Department of Clinical Biochemistry, John Radcliffe Hospital, Oxford.

4.3 Results

4.3.1 Total Protein concentrations

There was a good correlation between the automated (COBAS MIRA Plus) and the manual (BCA) methods used to determine the Total Protein concentrations for each of the donor site wound fluid and burn wound fluid samples as shown in Figure 33 ($r=0.95$).

Figure 33 Correlation between the automated and manual Total Protein measurements in donor site wound and burn wound fluid samples



All the following Total Protein results displayed were those calculated using the manual BCA technique.

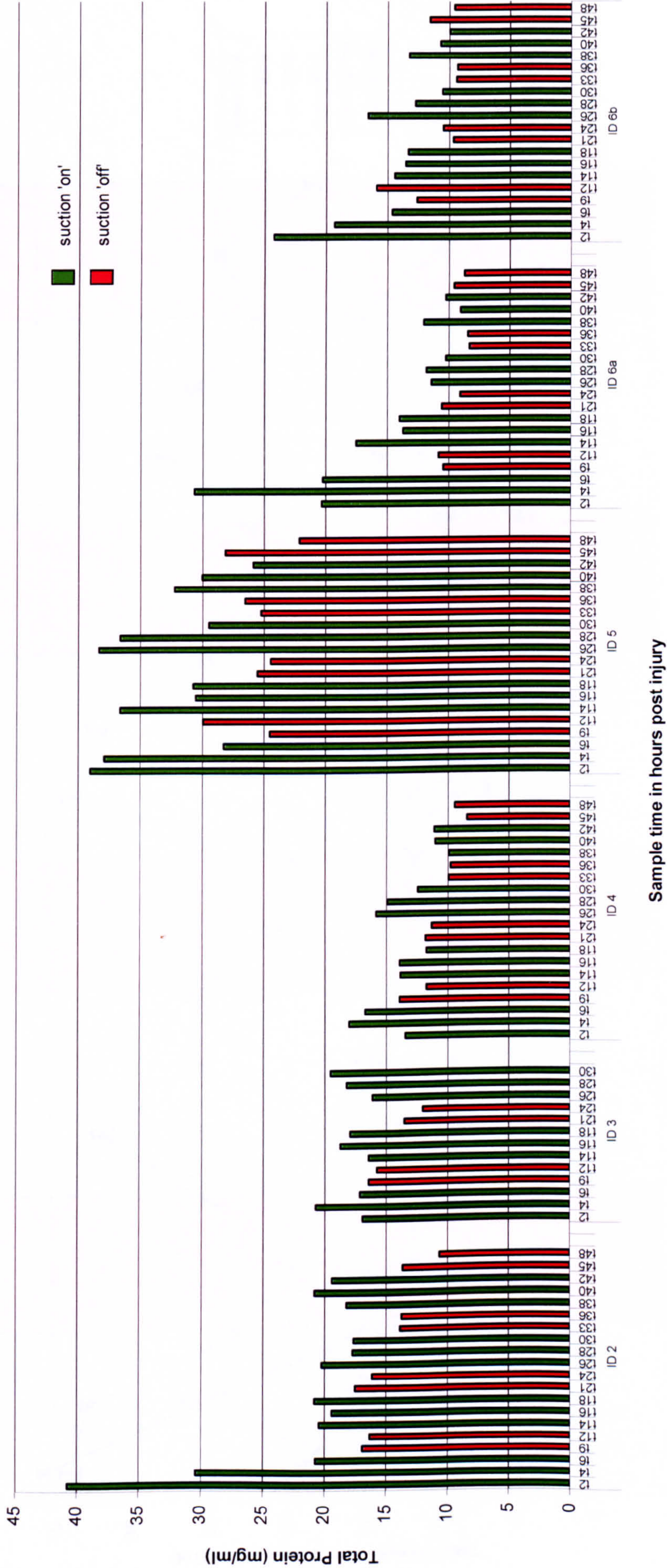
4.3.2 Effects of 'on/off' TNP therapy on Total Protein in donor site wound fluid

The results of intermittent TNP therapy on Total Protein concentration from donor site wound fluid collected using the small irrigation TNP device are shown in Figure 34. These same results are also displayed together in Figure 35.

The following observations can be made:

1. In each of these donor site wound fluid samples, Total Protein gradually falls from t2 hours throughout the collection times to t48, despite cyclical TNP therapy ($P < 0.0001$).
2. Intermittent (6-hourly) TNP therapy influences the concentrations of Total Protein throughout each set of donor site samples. During the 'on' suction phase, an increase in Total Protein is recorded. When TNP therapy stops, a fall in Total Protein is measurable (differences between 'on' and 'off' phases in each set of samples, $P < 0.0001$).
3. Within each 6-hourly period, there appears to be a trend of decreasing Total Protein values for each of the three consecutive samples collected during the 'on' suction phase, and for each of the two consecutive samples during the 'off' suction phase (not significant, NS). The sudden changes in Total Protein values occur most noticeably between the change in 'on' and 'off' cycle periods.
4. Total Protein concentrations can vary between patients as noted in the case of donor site ID5. Interestingly, donor site ID2 and ID5 wound fluid samples were collected following skin grafting procedures with the dermatome set at 0.01 inch whilst in the other donor site cases, the dermatome was set at 0.008 inch (see Table 9 on page 114).

Figure 34 Total Protein concentration in six donor site wound fluid samples under the influence of intermittent TNP therapy.



Each green and red bar represents the mean of three measurements. Standard errors of the mean not shown.

Figure 35 Total Protein concentration in six donor site wound fluid samples with reference to the on/off phases of TNP therapy. (Standard error of the means are displayed)

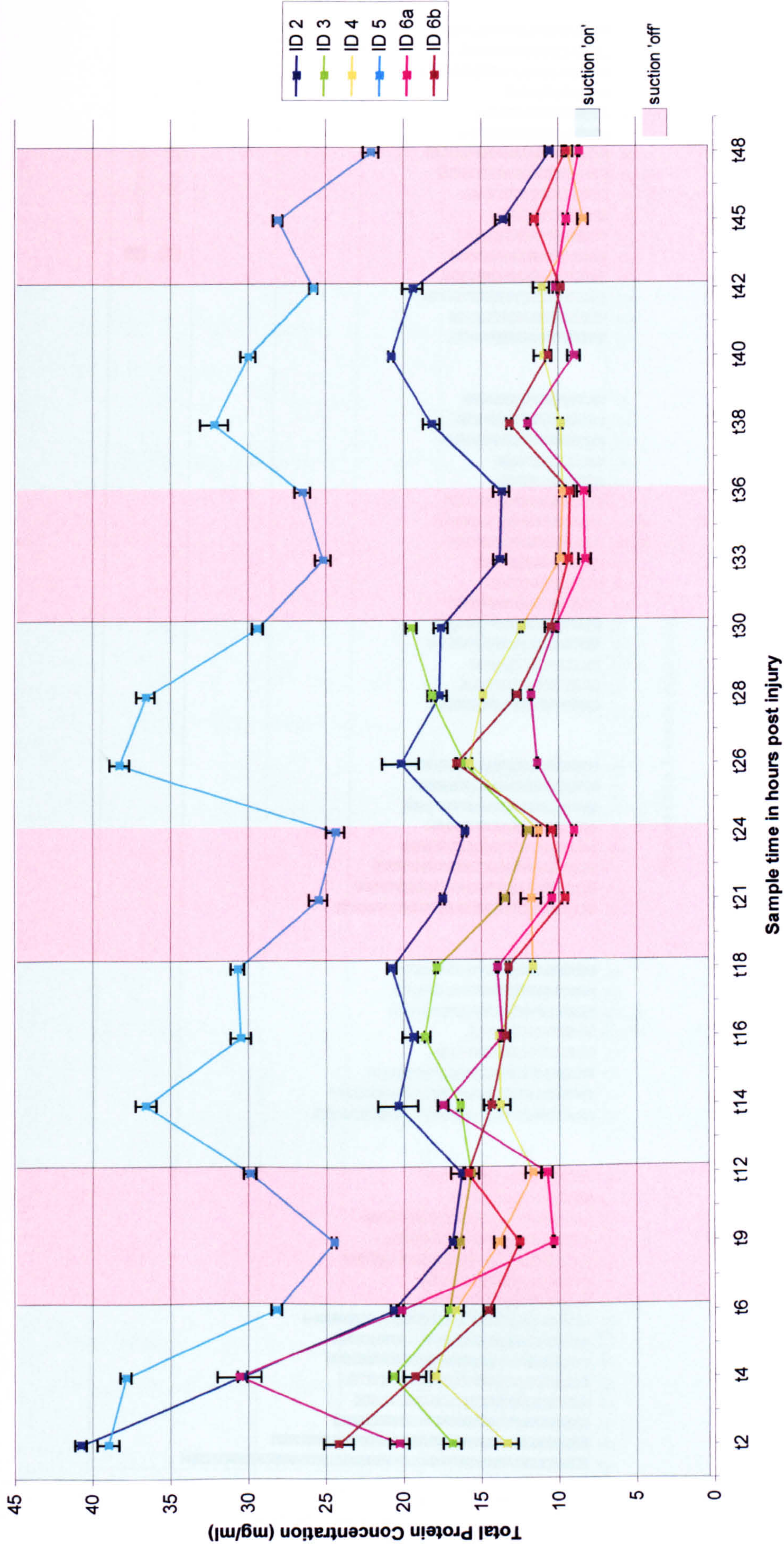
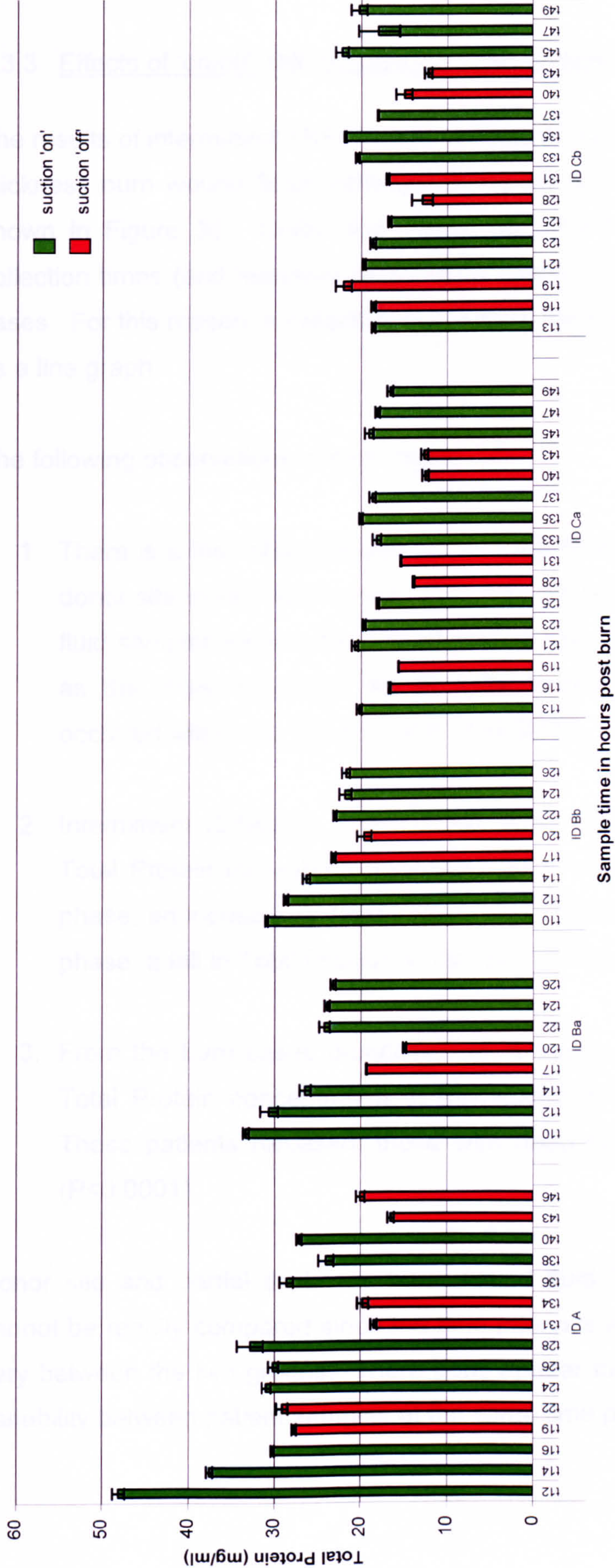


Figure 36 Total Protein concentration in five partial thickness burn wound fluid samples under the influence of intermittent TNP therapy.



Each green and red bar represents the mean of three measurements. Standard errors of the mean are displayed

4.3.3 Effects of 'on/off' TNP therapy on Total Protein in burn wound fluid

The results of intermittent TNP therapy on Total Protein concentrations from partial thickness burn wound fluids collected using the small irrigation TNP device are shown in Figure 36. Unlike the donor site wound fluid samples, the start of collection times (and therefore on/off cycle times) vary between burn wound fluid cases. For this reason, a collective overview of the burn data can not be displayed as a line graph.

The following observations can be made:

1. There is a less characteristic fall in Total Protein concentration than in the donor site wound fluid cases. This may be due to the fact that burn wound fluid samples were not collected until as late as t10 hours post burn whereas the most significant fall in Total Protein in donor site wound fluid occurred within the first six hours ($P<0.0001$).
2. Intermittent (6-hourly) TNP therapy does influence the concentrations of Total Protein throughout each burn patient case. During the 'on' suction phase, an increase in Total Protein is recorded, and during the 'off' suction phase, a fall in Total Protein is recorded ($P<0.0001$).
3. From the burn cases demonstrated in Figure 36, there is slightly greater Total Protein concentration in the wound fluid samples of A, Ba & Bb. These patients represent those with deep partial thickness burn wounds ($P<0.0001$).

Donor site and partial thickness burn wound fluid Total Protein concentrations cannot be readily compared since the time intervals at which samples were taken vary between the two groups. There does appear to be a degree of inter-patient variability between patient samples at the same time points in similar wounds.

Despite this, overall concentrations of Total Protein are greater in the burn wound fluid cases compared to the donor site wound fluid cases for given sample times ($P<0.0001$).

4.3.4 Comparing volumes measured during collection against Total Protein

Since the results above strongly suggest that the concentration of Total Protein is greater during the 'on' suction phase of TNP therapy than during the 'off' phase, it is possible that these significant observations may be due to the nature of the fluid collection technique during these phases. For example, there might be a dilutional effect in the method of sample fluid retrieval during 'off' phase, which might account for the measured differences between 'on' and 'off' phases.

To establish whether larger fluid volumes from each sample retrieved during the collection method were linked to a reduction (dilution) in Total Protein levels, a scatter plot was constructed to demonstrate the volume of each wound fluid sample versus the measured Total Protein concentrations, for the entire donor site samples and then for all the burn samples.

Figure 37 demonstrates that there is no evidence of a correlation between the volume of fluid collected and the concentration of Total Protein measured over a 1-minute collection time (correlation coefficient = 0.047). Of 2mls Normal Saline irrigant fluid, approximately 75% is commonly retrieved over this time (mean=1.59mls; SD= 0.61; n=93 samples). This is in keeping with the fluid volume measurement tests carried out on normal skin (see Table 8 on page 105). Furthermore, there is no association between Total Protein and volume of fluid collected during the 'on' versus 'off' phases ($P=0.12$).

Figure 38 also demonstrates that in burn wound fluid collections, there is no correlation between volume and the concentration of Total Protein measured in the samples collected over a 1-minute period (volume mean=2.05mls; SD=1.14; n=63 samples, correlation coefficient = -0.114). One isolated value of 7mls measured on one occasion might account for the larger standard deviation (SD). This particularly large value was due to a failure to ensure that the sterile collection

container cap was secure. Subatmospheric pressure leaked from the system and when recognised and corrected, the pressure in the pump (that had increased to compensate for the leak) resulted in a transient and sudden increase in fluid sucked up through the TNP device over the 60-second collection period. Again, there is no association between Total Protein and volume of fluid collected during the 'on' and 'off' phases ($P=0.62$).

Figure 37 Scatter plot of Total Protein concentrations against volume of sample in donor site wound fluid collections over 1 minute (n=93 samples; $R=0.047$)

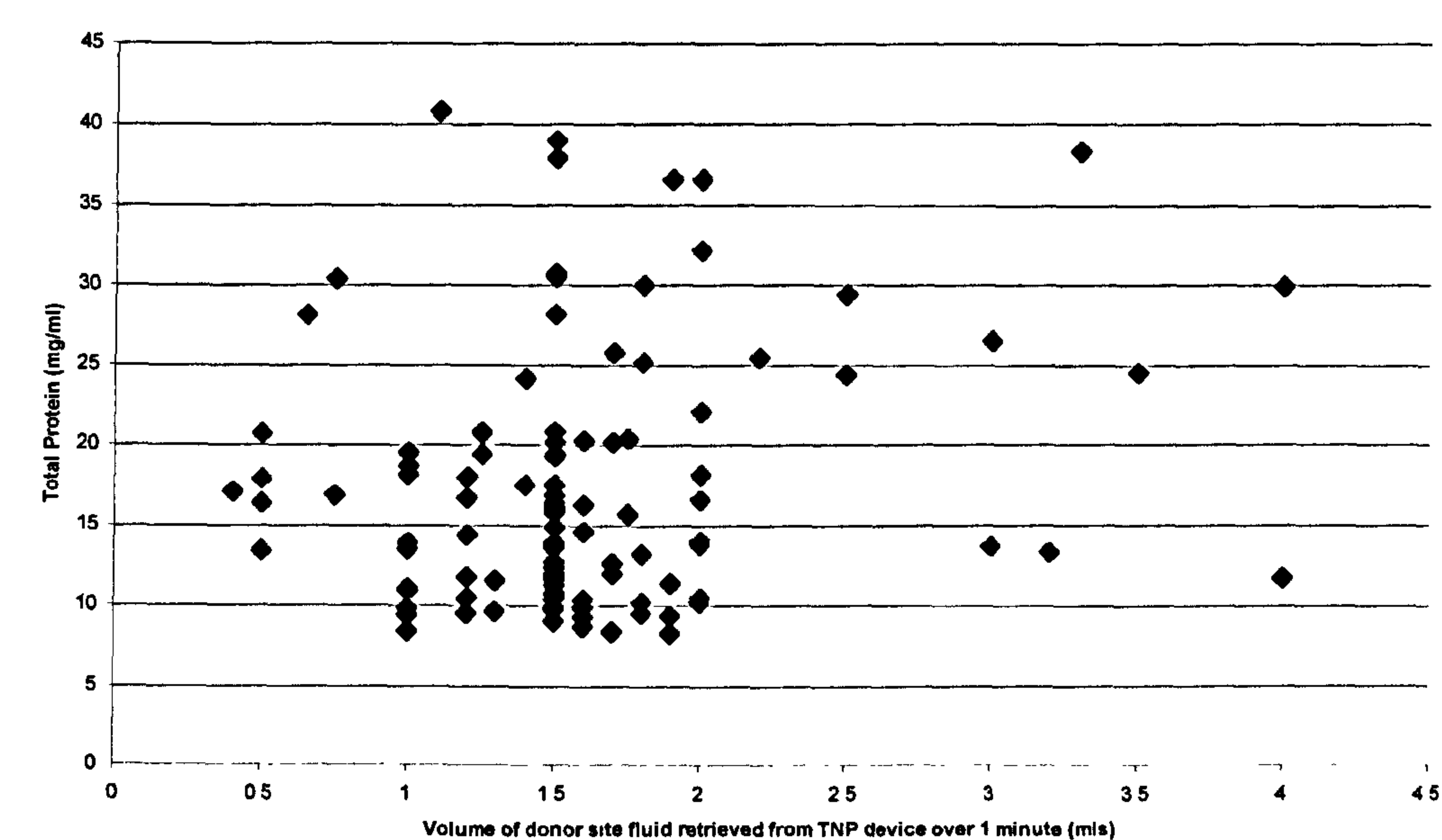
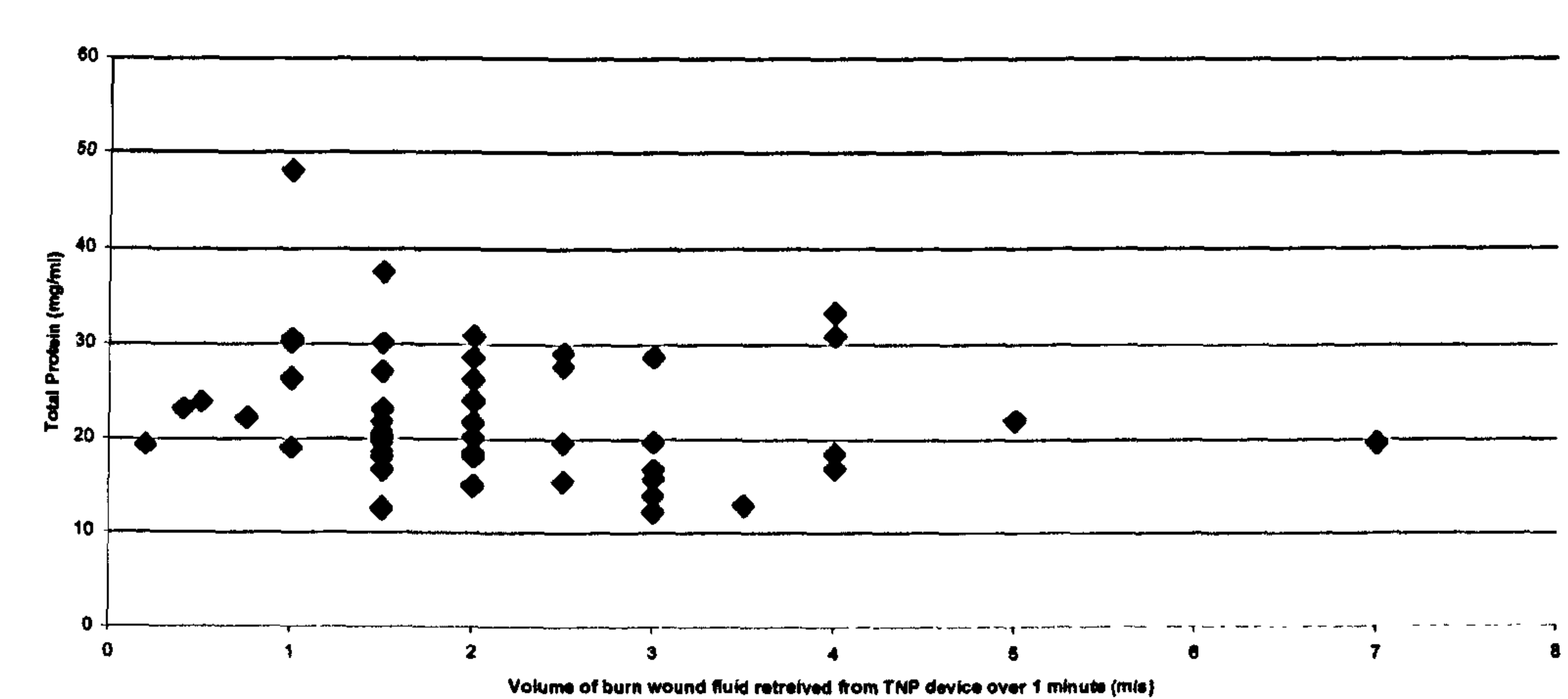


Figure 38 Scatter plot of Total Protein concentrations against volume of sample in burn wound fluid collections (n=63 samples; $r=-0.114$)



4.4 Discussion

4.4.1 TNP in the modulation of acute human cutaneous wounds

This study has clearly demonstrated that TNP therapy is capable of modulating the dermal wound fluid environment in both acute donor site and burn wounds in humans as determined by measuring Total Protein in samples retrieved from the wound surface during on/off TNP suction cycles.

There were a number of reasons for using the iatrogenic donor site wound as a model to assess the effects of TNP therapy on wound healing. Firstly, the donor site wound is readily available following skin grafting procedures in plastic surgical departments. Secondly, the wound can be easy to create uniformly using a mechanical air-driven dermatome on a readily accessible thigh in most cases. Thirdly, Genecov et al. (1998) have used TNP safely on the donor site wound in both pigs and humans for up to 14 days to assess rates of reepithelialization.

Human partial thickness burn wounds represent a distinctly different acute dermal injury. TNP has been mentioned as a possible treatment for burns in humans (Morykwas and Argenta, 1997). TNP therapy has been applied to experimentally induced partial thickness burn wounds in a swine model for up to 48 hours to demonstrate that it might be a useful modality in the prevention of burn wound progression (Morykwas et al. 1999a).

This study also utilised TNP therapy on donor site and burn wounds for a period of 48 hours. However, the intention in this study was not to assess the efficacy of TNP directly on clinical wound healing, but to determine if TNP might be able to alter the fluid environment of wounds, which might be attributable to one of its mechanisms of action.

Follow-up of these patients after this treatment at 2-3 weeks was performed to ensure that each patient was happy about their healing wounds and to exclude complications that might have been caused by this TNP device. No complications, such as pressure effects that might have been created by the rim of the device or

infection, were noted in these cases. No attempt was made at this time to measure rates of healing in terms of reepithelialisation, for example. There was no subjective evidence for any differences in the TNP-treated and control-treated wounds noted at any stage post-operatively.

4.4.2 Correlation between Total Protein concentrations and fluid volumes

There was no evidence to suggest that the changes in Total Protein concentrations measured during on/off suction might be accounted for by any differences in the method in which the samples were collected during the 'on' or 'off' periods. Since the majority ($\approx 75\%$) of the irrigant fluid is sucked up within the 60 second collection time, any observed differences in Total Protein concentration measured is less likely to do with a dilutional effect of excess irrigant fluid in the device during the 6-hourly 'off' period. There was no evidence that greater wound fluid volumes were matched by a decrease in Total Protein concentration using this device. The differences in on/off periods measured here are more likely to be the direct result of subatmospheric pressure drawing proteins from the wound itself. This was graphically illustrated in Figures 35 and 36.

The donor site wound creates an open, blood filled injury with direct damage to the dermal circulation with leakage of blood directly onto the denuded skin surface. The partial thickness burn wound is a 'blood-sealed' exudative wound. Despite this, there appeared to be more wound fluid retrieved during TNP therapy on burn wounds than donor site wounds during the collection times, with statistically significant increases in Total Proteins measured in burn wound fluid samples than donor site wound fluid samples in this study. Although, the method of collection was different, the total proteins measured in this study are consistent with those of Rao et al. (1995), who measured total protein of between 15-40mg/ml of acute donor site wound fluid.

4.4.3 TNP irrigation device as a research tool in wound fluid collection

The use of Normal Saline as an irrigant medium to wash the surface of a wound and to allow collection of wound fluid has been employed by others (Breuing et al., 1992; Vogt et al., 1998). Vogt et al. used open wound chambers mounted on similar silicone sheets directly on human donor site wounds following split-skin graft excision, and filled them with 2.5mls of Normal Saline. After 24 hours, the total fluid (between 2-4mls) was aspirated and replaced with a further 2.5mls. Samples were collected every 24 hours using this method over an 11-day period. In their wound fluid samples, total protein concentrations dropped from an average 5.5mg/ml on day 1 to a baseline of 0.1mg (unwounded skin), indicating a return to barrier function. Using the irrigation TNP device employed in this study, 2mls of Normal Saline was instilled for 1 minute only, directly onto the wound surface and removed over a subsequent 60 seconds before being centrifuged and stored. By day 1, in this study, total protein concentrations varied from 9.03-24.4mg/ml. The total protein in this study fell significantly over 48 hours. Exact comparisons of total protein concentrations cannot be made with the results of Vogt et al., since the method of dilution and wound fluid extraction differ. However, it is possible that the greater total protein concentrations measured in this study using 0.5mls less Normal Saline and collected over 1 minute as opposed to 24 hours, may be accounted for by the use of suction therapy. This suggests that TNP therapy might actively remove total protein from the wound.

The advantage of this irrigation TNP device is that it allows for frequent 'fresh' sampling of wound surfaces every two or three hours. Extraction of biologically active wound fluid might be achieved almost instantly, without the problems of delay between collection and freezing prior to analysis (see chapter 6). The time from instillation of the irrigant to freeze storage takes less than 10 minutes in this study. Hence, this device may provide a potentially useful tool for the collection of wound fluid for immediate analysis in cutaneous wound healing research, which has not been described before in the literature (see section 1.4.4).

Unfortunately, this study did not examine whether proteins or other factors might be trapped within the foam dressing during suction. There has been no evidence

reported in the literature that the foam dressing is capable of trapping proteins or extracellular matrix factors directly. It is possible that the results shown in this study reflect differences in proteins sticking to the foam. Despite this, it is not likely that during the two 3-hourly off phase sampling times, sufficient sticking of protein to the foam would account for the reduction in protein concentrations measured. Importantly, during these 'off' collection times, the suction is switched on transiently (1 minute) to extract the fluid sample, and it might be expected that any trapped proteins might be released with the irrigant fluid during this time.

It is possible that the increases in protein concentrations measured during the 'suction-on' phase, may be attributable to the increase in blood flow, which has been demonstrated both in this thesis (chapter 3, section 3.3.3.2) and by Banwell et al. (2000) using laser Doppler imaging. The mechanical effects of suction through the foam interface is likely to explain the measured increases in protein concentrations.

There are a number of other possible reasons for variations in measurements obtained in this study. These might include the age and sex of each patient, the depth of the burn or dermatome depth and time from burning, in addition to differences in the systemic effects of burns or haematological variations. Nonetheless, each patient wound acts as its own control, which makes comparisons between the on and off suction phases more straightforward. Care was taken to perform each set of experiments over the same time period or within a 48-hour burn period. The method of fluid sampling was the same.

A valid criticism of this study concerns the interpretation following wound irrigation. It became apparent during the study designs in chapter 3, that irrigation was necessary in order to provide enough quantity of fluid for sampling, and that pooling of fluid within the chamber was possible. Although the method of fluid retrieval was the same, the observed reductions in protein concentrations obtained during the off-phase may be due to a dilutional effect from the build up of irrigant fluid from the previous sampling. Unfortunately, the exact volume of fluid retrieved over the 1-minute collection time was not equal to the 2mls of irrigant fluid placed into the chamber prior to each sampling. As Figure 37 and Figure 38 show, the amounts retrieved were variable. This variation might be due to previous build up of fluid within the chamber. This might alter the relative concentrations of protein

during each collection time, which is dependent on the timing of the previous collection within either the on or off phase.

4.4.4 The potential effects of TNP on Total Protein in other wounds

It has been well documented that TNP therapy provides a useful alternative in the management of chronic wounds. Falanga and Eaglstein (1993) proposed the 'trap' hypothesis in which macromolecular proteins (including albumin and α_2 -macroglobulin) might contribute to the development and maintenance of venous ulceration by the persistent leakage of these proteins into the dermis which they hypothesised might bind to or 'trap' growth factors and matrix material, making them unavailable for tissue repair (Falanga and Eaglstein, 1993). With this in mind, the use of TNP therapy in chronic dermal wound healing might be of advantage by actively removing such proteins from the wound and thus increasing the bioavailability of endogenous growth factors to wound healing.

4.5 Conclusion

This study has determined that the irrigation TNP device, which mimics V.A.C.™ dressings, modulates the wound fluid constituents of cutaneous wounds by actively removing extracellular proteins under subatmospheric pressure. Indirectly, this might explain one of the possible mechanisms by which TNP therapy promotes healing. It is likely that the increased levels of protein measured during the suction phase is related to the direct mechanical effects of suction on the wound fluid and its constituents, enhanced by the increased blood flow that is present in the wound under the TNP device.

The irrigation TNP device employed in this study has been used safely on human dermal wounds and might be a useful research tool in collecting dermal wound fluid from a number of acute and chronic wounds for temporal analysis in wound healing studies.

The ability to apply selective Topical Negative Pressure therapy to a wound, provides an opportunity to compare the cells of treated and control wounds more closely. This is investigated in chapter 5.

If some of the constituents of the wound fluid (such as proteases, which are known to be present at increased levels in difficult-to-heal wound fluids) are also modulated by TNP therapy then this might explain one possible mechanism of action of this type of therapy. This hypothesis is explored in chapter 6.

5 Histological investigation into the effects of Topical Negative Pressure therapy on neutrophils and their distribution in partial thickness dermal wounds

5.1 Introduction

Applying selective TNP therapy to partial thickness dermal wounds in human subjects provides an opportunity to identify cellular differences that might occur between TNP-treated and control sites within each patient.

This observational study was conducted in order to establish whether TNP therapy is able to modulate the number of neutrophils and their temporal distribution within the dermis of open human dermal wounds over 48 hours post-injury, compared to untreated controls. An immunohistological stain using a specific monoclonal antibody (NP57) to Neutrophil Elastase (NE), was used to identify neutrophils more clearly for cell counting, as well as to identify areas in which degranulation of NE from neutrophils may be occurring.

5.2 Materials and Methods

5.2.1 Study design

A longitudinal intra-patient controlled histological study was performed on biopsy samples taken from patients with donor site wounds undergoing TNP therapy over the first 48 hours following injury. A similar study was performed on biopsy samples taken from partial thickness burn wounds undergoing TNP therapy from the time of admission up until 48 hours following burning.

5.2.2 Patient selection

Tissue biopsies were collected from six donor site wounds (n=6 patients) and six partial thickness burn wounds (n=4 patients). Four patients who participated in the wound fluid studies earlier, also consented for biopsies of their wounds. Two further patient biopsy samples were obtained from two further donor site patients (ID 8 and ID 9). Patients selected for this study are identified in Table 12 below.

The same burn cases used in the previous studies were also used in this study, supplemented with a further case (ID F). See Table 13 below. The differences in TNP management between burn F and the other burn cases was that TNP therapy was applied continuously, without 6-hourly on/off cycles.

Table 12 **Patient details of donor site biopsy sites and times**

ID	Age	Sex	Donor site	Control Biopsy t0	Control biopsy t48	TNP biopsy t48
1	74	F	Right thigh	in theatre	at bedside	at bedside
2	42	M	Right thigh	in theatre	at bedside	at bedside
4	19	F	Right thigh	in theatre	at bedside	at bedside
5	32	F	Right thigh	in theatre	at bedside	at bedside
8	20	M	Right thigh	in theatre	at bedside	at bedside
9	64	F	Left thigh	in theatre	at bedside	at bedside

Table 13 **Patient details of partial thickness biopsy sites and times**

ID	Age	Sex	Burn site	Burn depth	Admission biopsy	Control biopsy	TNP biopsy
A	44	M	Right chest	DPT	t12	t46	t46
Ba	19	F	Right buttock	DPT	t10	t30	t30
Bb	19	F	Left buttock	DPT	t10	t30	t30
Ca	26	M	Left leg	SPT	t16	t49	t49
Cb	26	M	Left leg	SPT	t16	t49	t49
F	18	M	Left arm	SPT	t3	t48	t48

DPT – Clinical deep partial thickness burn (required surgery)
SPT – Clinical superficial partial thickness burn (healed within 3 weeks)

5.2.3 Collection and storage of biopsy specimens

The following procedure was used to obtain donor site wound biopsies.

In the operating theatre, immediately after skin graft harvesting, a 4mm-diameter punch biopsy (Stiefel, Germany) of dermis was taken of one area of the donor site wound from each patient. See Figure 29 on page 116. The biopsies were placed into a square basket, and orientated length ways along its side to prevent distortion of the specimen. The basket was placed directly into 4% neutral buffered formaldehyde ((Cellstor® Pot, CellPath Ltd., UK.).

Following application of the TNP irrigation device adjacent to the non-biopsied area of donor site wound, the surrounding control wound was dressed using standard dressings (Kaltostat™, cotton gauze and crepe) in theatre.

After 48 hours, on completion of the last 48-hour wound fluid sample collection, the irrigation device and control dressings were removed from the wound for inspection. A further series of biopsies were taken under local anaesthetic; one from the control-treated wound (not less than 1.5cm from the previous biopsy site and one from directly under the TNP-treated site. The biopsy specimens were processed as described above.

2mls of 1% plain lignocaine was used to surround each biopsy site prior to harvesting, with infiltration under and not within the dermis, to reduce any possible artifactual errors imposed by the infiltration of a volume of fluid within the dermis.

Each biopsy site was sutured with interrupted 5/0 Prolene, and the entire donor site wound was then redressed with standard Kaltostat™, gauze and crepe dressings.

The following procedure was used to obtain partial-thickness burn wound biopsies.

Following clinical assessment of the patient's burn wounds on arrival in the Burn's Unit at Stoke Mandeville Hospital, biopsy specimens were taken from partial-thickness burn sites at the time of admission under local anaesthetic as described above.

Intermittent TNP therapy was commenced on non-biopsied contiguous burn wound areas and wound fluid collected up to 48 hours as described in Chapter 4

(except for burn F in which continuous suction was applied). Standard Bactigras™ and gauze dressings were placed on adjacent burn wound sites.

After the last 48-hour wound fluid collection using the irrigation TNP device, all the dressings were removed and the burn wound surface examined. A further two biopsy specimens were taken from control and TNP-treated burn sites and processed in the same fashion as described above, under local anaesthetic. An example of these biopsies can be seen in Figure 27 on page 107. The entire burn wound was redressed in the same standard burn dressings as described for the control sites.

5.2.4 Formaldehyde fixation and sectioning

After 24 hours, each biopsy was transferred (within its basket) to an automated machine (Shandon Scientific Ltd., UK.) for further 10% formaldehyde fixation for 5 hours prior to graded dehydration in Industrial Methylated Spirit (IMS) at 70%, 85%, 90%, and three lots of 100% for 1 hour each. This was followed by two lots of 1-hour xylene and three lots of 1-hour paraffin waxing before being embedded in a block of paraffin wax for sectioning.

For immunohistochemical staining, 3µm thick sections were cut (Anglia Scientific microtome) from each biopsy block and between 2-3 sections placed onto SuperFrost® Plus (Menzel–Glaser, Germany) slides. These slides were dried and stored at room temperature prior to staining for Neutrophil Elastase.

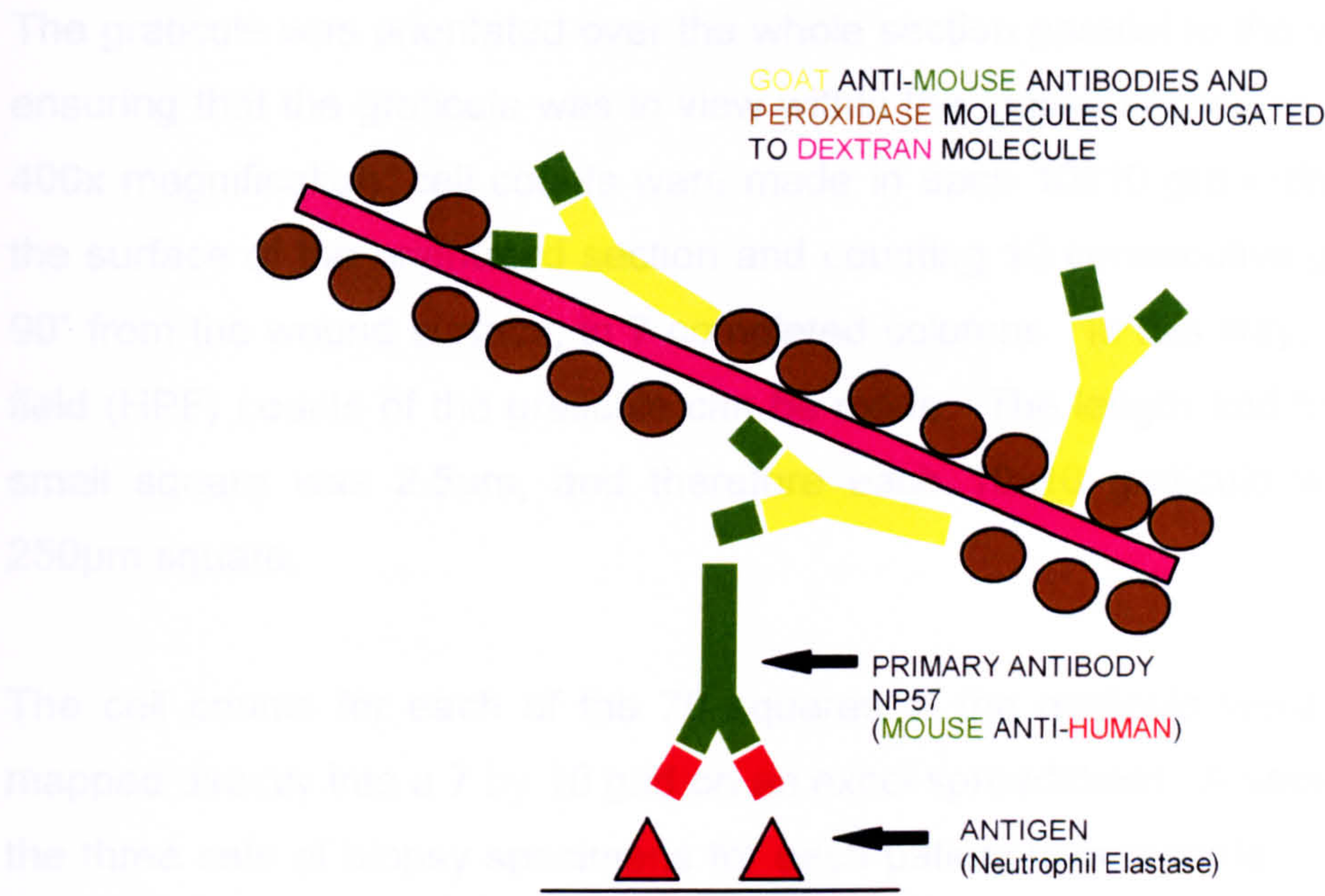
5.2.5 Immunohistochemistry for Neutrophil Elastase

The use of the mouse anti-human neutrophil Elastase primary monoclonal antibody, NP57 (Dako, UK.), was chosen as both a marker for neutrophils, in order to aid cell counting, and to assess the extent of neutrophil degranulation of Elastase from the intracellular azurophilic granules. The primary antibody, in turn, was labelled with a highly sensitive, commercial system, EnVision™+, based on a peroxidase labelled dextran polymer conjugated to goat anti-mouse

immunoglobulins. Further incubation with a 3,3'-diaminobenzidine (DAB) chromogen solution results in a brown-coloured precipitate at the antigen (Neutrophil Elastase) site. See Figure 39 below.

Neutrophil Elastase (NE) was detected in neutrophils using the EnVision™+ system. The primary antibody was NP57 (mouse anti-human NE) and the secondary antibody was EnVision™+ (goat anti-mouse IgG).

Figure 39 The indirect immunohistochemical staining technique with the EnVision™+ system



The EnVision™+ reagents were purchased as a kit (K4006, Dako, UK) and used in the immunohistochemical protocol as described in Appendix 10.4.

All immunohistochemical staining of biopsy sections taken from donor site wounds were performed in one batch, and repeated for the biopsy sections taken from partial thickness burns. Human tonsillar tissue and one spare patient test slide of human skin were used as positive and negative control tissue respectively, to validate the staining technique in each batch. The use of PBS was used to replace the primary antibody as the negative control reagent.

5.2.6 Neutrophil counting technique

A representative section of each biopsy was chosen for neutrophil cell counting. Neutrophil Elastase-stained neutrophils were quantified in the section by manual cell counting of the brown cells under a Zeiss light microscope, using a 10x10 square graticule chamber placed in one eyepiece.

The graticule was orientated over the whole section parallel to the wound surface, ensuring that the graticule was in view within the biopsy section at low power. At 400x magnification, cell counts were made in each 10x10 graticule, starting from the surface of the orientated section and counting 10 consecutive grid squares at 90° from the wound surface, in 7 completed columns. In this way, 70 high power field (HPF) counts of the graticule can be made. The length and breadth of each small square was 2.5µm, and therefore each 10x10 graticule was 250µm by 250µm square.

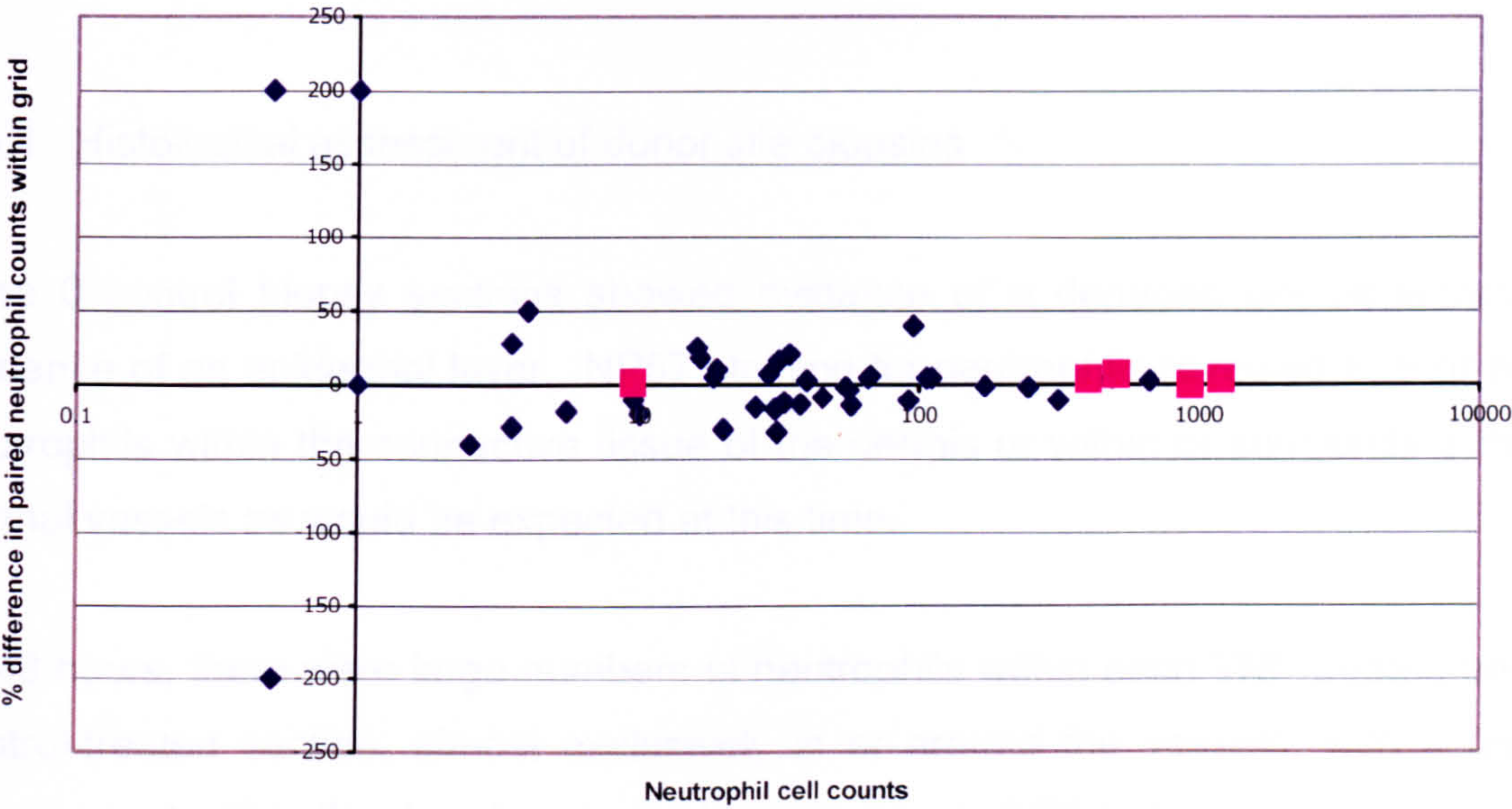
The cell counts for each of the 70 squares of the graticule were recorded and mapped directly into a 7 by 10 grid on an excel spreadsheet. A section for each of the three sets of biopsy specimens for each patient was recorded. In this way, a large representative number of cells can be counted in defined areas of the section, thus providing an indication of the distribution of cells within the section from the surface of the wound and down into its depth.

The author, using the same light microscope, performed cell counts blind. Details of these counting grids are displayed in Appendix 10.5 and 10.6.

5.2.7 Reproducibility of counting technique

Reproducibility of the counting technique was assessed by the blind repetition of cell counting for a random sample of 5 biopsy sections. An Altman-Bland plot was constructed as shown in Figure 40 below.

Figure 40 Altman-Bland plot of neutrophil counts of 5 random biopsy sections, using the method of counting as described in the text. No significant differences were seen between the paired counts taken at two different times. (Correlation = 0.99)



This graph highlights that each of the paired values (n=50, diamonds) obtained within the grid at various levels measured from the surface of the wound correlate very well when repeat counts were performed ($R= 0.99$). The mean paired neutrophil counts for each of the five random sections measured are shown by squares. There is no significant difference between the % difference in paired neutrophil count values, which were counted on separate occasions. This procedure supports the reproducibility and accuracy of the cell counting technique as a means of determining neutrophil numbers and their distribution within the biopsy sections.

5.3 Results

The following results display histological differences in neutrophil numbers and their distribution within wound biopsies between TNP-treated and control sites as identified by the NP57-positive stained cells. Examples of the immunohistochemical stains can be seen in Figure 41 and Figure 42 on page 146.

5.3.1 Histological assessment of donor site biopsies

Time 0 control biopsy sections showed evidence of a denuded dermis without evidence of an epidermal layer. NP57 staining for neutrophils revealed little or no neutrophils within the connective tissue of the dermis or within or surrounding the dermal vessels as would be expected at this time.

At 48 hours, there were large numbers of neutrophils within each TNP-treated and control-treated section, almost exclusively in or around the vessels, with a few interspersed within the dermis proper. In some cases, NP57 staining was seen as 'clouds' within the sections accompanying ghost-like neutrophils consistent with degranulation, although no clear differences could be seen between the TNP-treated and control sections. Occasionally, some sections were stained with fibrin clot on the surface of the wound. Within the clots, large numbers of red blood cells (shown clearly on the PAS sections) and NP57 positive cells were seen. The demarcation between the clot and the surface of the wound was clearly defined. Cell counts from these sections were only performed from immediately under the surface, avoiding the NP57 positive cells within the clot.

The most striking differences were noted in the neutrophil counts within the dermis between the paired intra-patient biopsy sections at 48 hours. There were fewer neutrophil numbers in the TNP-treated biopsy section compared to their intra-patient control-treated biopsy section taken at the same time in all six cases ($P < 0.002$). See Table 14 and Figure 43.

Furthermore, differences can be observed in the number of neutrophils between donor site wounds, suggesting a degree of inter-patient variability (see Table 14).

Table 14 **Average neutrophil cell counts per 70 HPF in donor site wound biopsies**

Donor site biopsy	t0 biopsy	t48 control biopsy	t48 TNP biopsy
ID 1	0.07	12.2	9.13
ID 2	0.21	10.63	6.27
ID 4	0.14	7.34	3.76
ID 5	0.16	17.71	9.66
ID 8	0.06	16.49	8.03
ID 9	0.36	25.81	4.76

5.3.2 Histological assessment of partial thickness burn wounds

Each set of burn wound biopsies differ between patients both in terms of the times at which they are taken, and also because of the nature and extent of the burn and its depth. Many more variables are present in these than the donor site wounds, making paired intra-patient comparisons more critical.

Neutrophils were observed in perivascular areas, with many more within the connective tissues of the damaged dermis compared to donor site wound biopsies. Heavy degranulation of neutrophils with scattered NP57 staining outside cells was particularly noticed at and near the surface of the sections. Observations of each biopsy section did not reveal any gross differences between the clinically superficial or deep partial thickness burn wounds with respect to neutrophil degranulation. Furthermore, no obvious differences in degranulation were seen between TNP-treated and control biopsies.

Biopsies taken at the time of admission into the Burns Unit varied from time 3 to 10 hours post burn. Average neutrophil numbers ranged from between 2.13 to 13.3 per 70 HPF section. See Table 15 below.

Comparing the paired 48-hour TNP-treated and control sections, the average neutrophil count per paired 70 HPF sections followed a similar pattern for that of the donor sites biopsy sections. Except in one case, (ID F), all the paired counts demonstrated a reduction in neutrophil counts in TNP-treated biopsy sections compared to their controls ($P<0.008$) as shown in Figure 44.

Table 15 Average neutrophil counts per 70 HPF in partial thickness burn wound biopsies

Burn site biopsy	Clinical burn depth	Biopsy timing (hours post burn)	Admission biopsy	Control biopsy	TNP biopsy
ID A	DPT	t10	3.96		
		t48		16.8	7.31
ID Ba	DPT	t5	10.3		
		t30		34.4	20.0
ID Bb	DPT	t5	12.4		
		t30		29.9	9.04
ID Ca	SPT	t10	2.13		
		t48		1.94	2.91
ID Cb	SPT	t10	5.99		
		t48		3.64	2.43
ID F	SPT	t3	13.3		
		t48		39.5	21.8

DPT – deep partial thickness
SPT – superficial partial thickness

Again, inter-patient differences in neutrophil counts were observed, especially in the superficial partial thickness burn cases.

There appeared to be a greater number of neutrophils in the clinically deeper partial thickness burns compared to the superficial partial thickness ones, although this is not statistically significant.

Figure 41 Section of NP57 positive-stained neutrophils (brown) in a burn wound at 48 hours. Note the preponderance of cells both around the vessels and near the wound surface

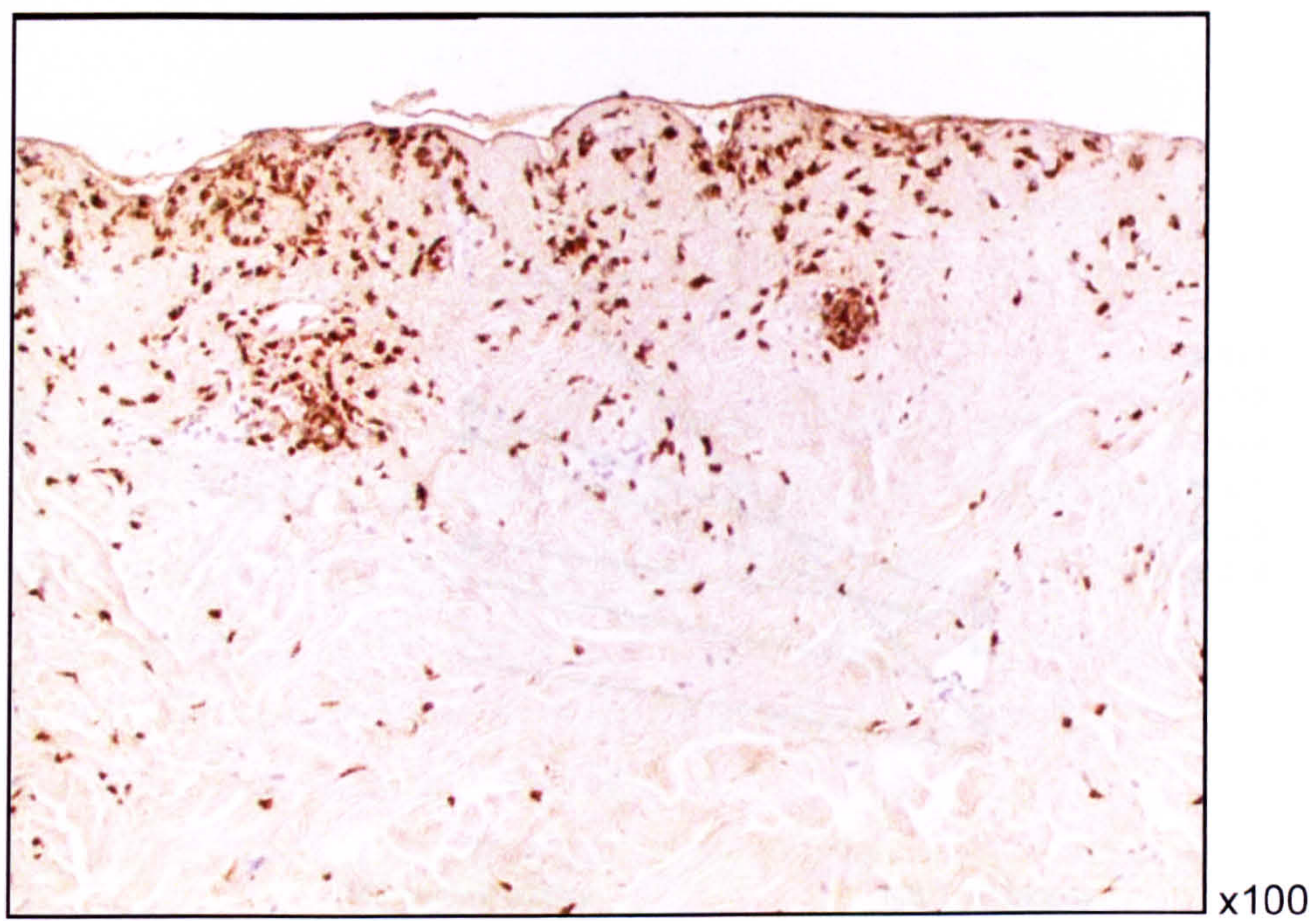


Figure 42 High-powered view of NP57-stained neutrophils. Extravasating neutrophils are degranulating beyond the vessel wall by 48 hours

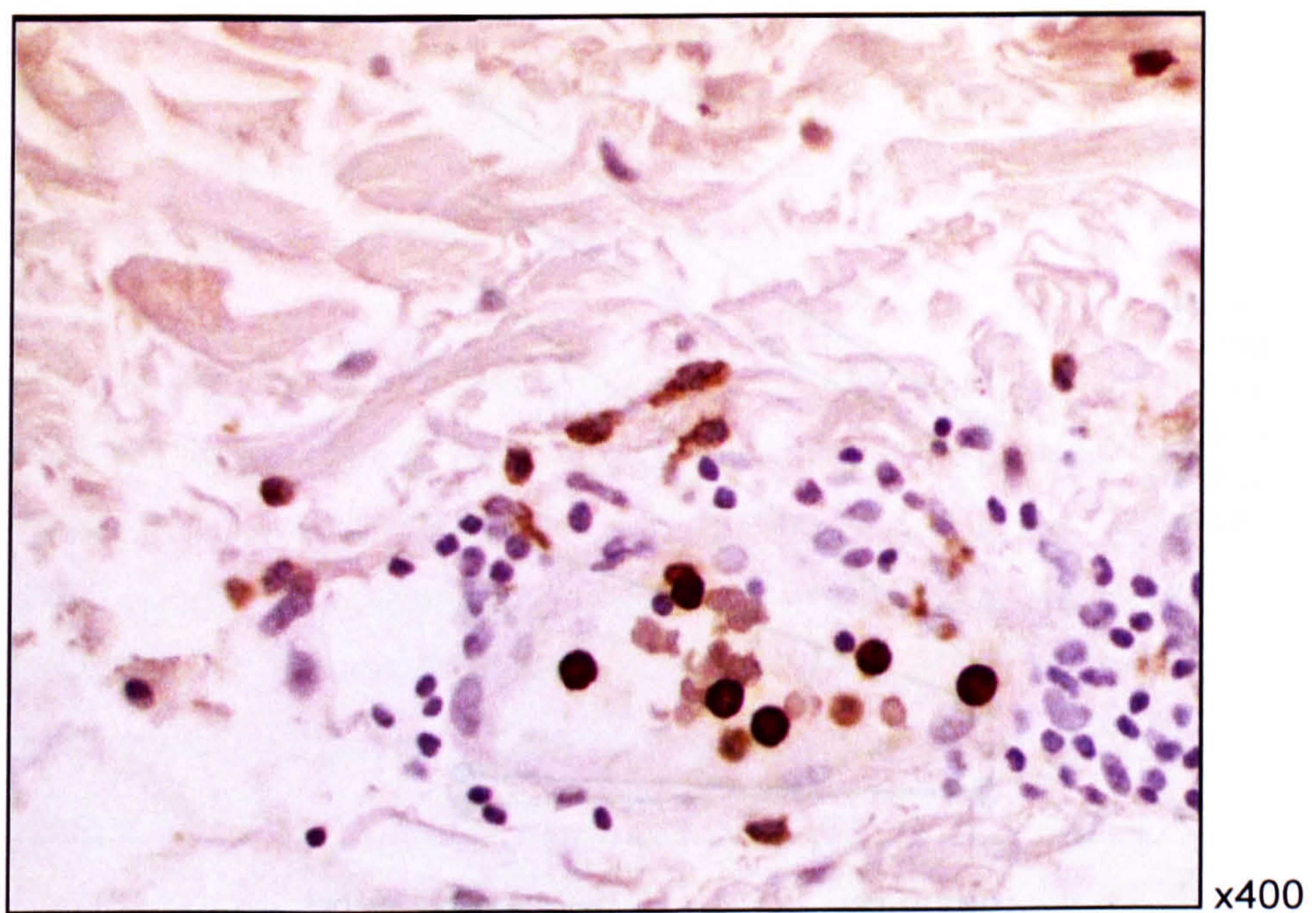


Figure 43 The effects of TNP therapy on neutrophil numbers within paired donor site wound biopsies at 48 hours post injury (n=6)

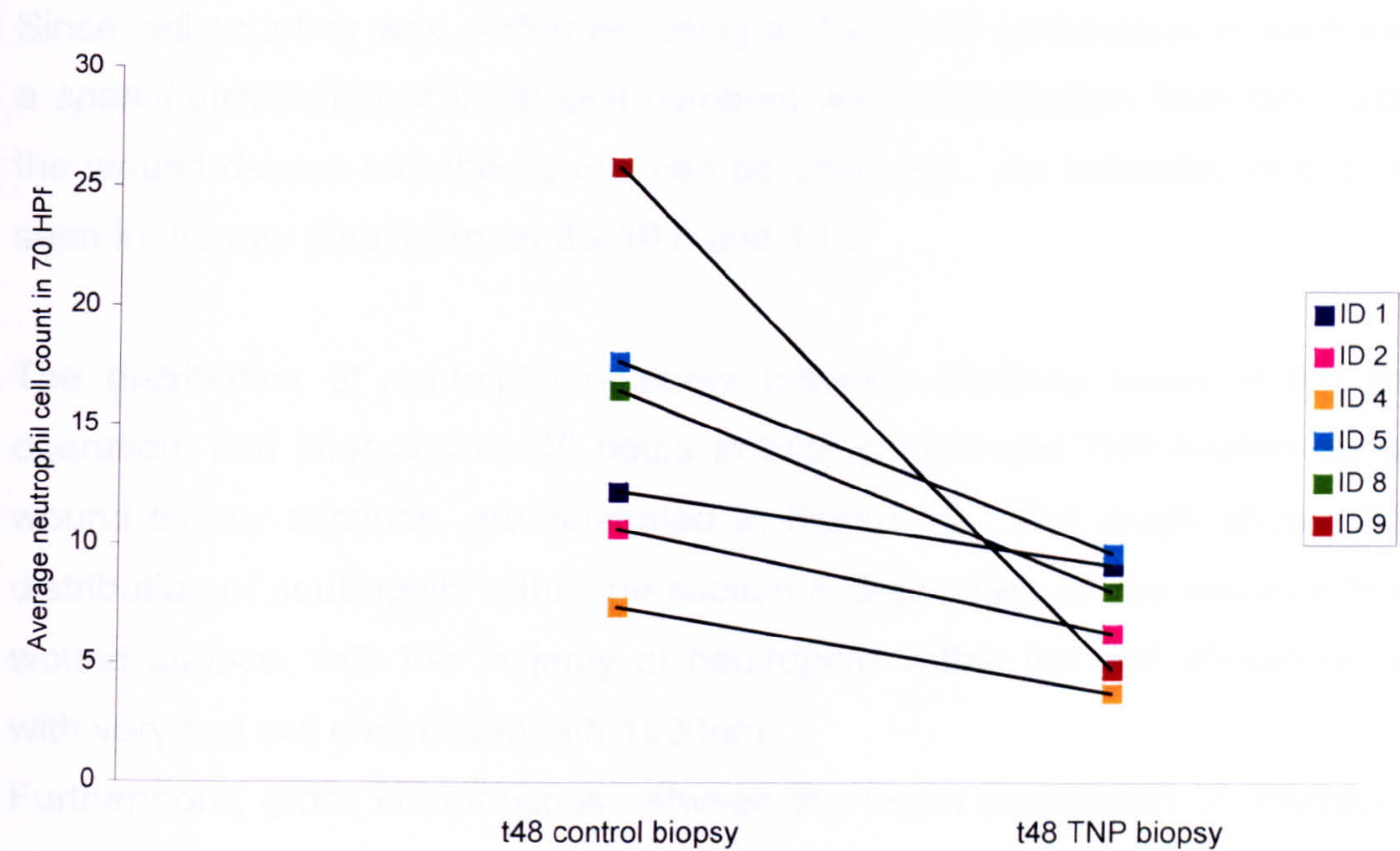
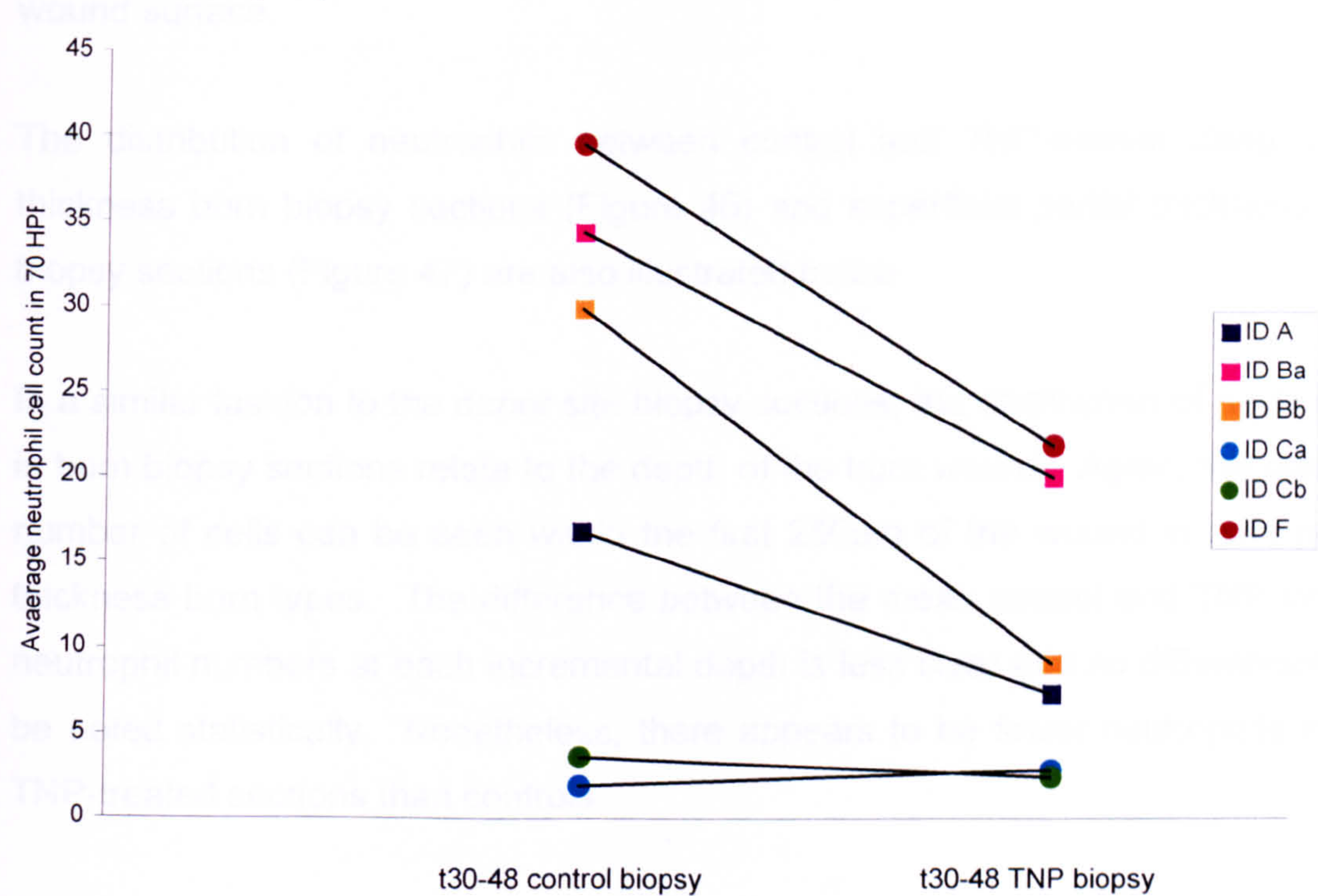


Figure 44 The effects of TNP therapy on neutrophil numbers in paired deep (squares) and superficial (circles) partial thickness burn wound biopsies at 30-48 hours post burn (n=6)



5.3.3 Histological assessment of neutrophil distribution within biopsies

Since cell counting was performed using a strict 7x10 grid pattern in each section, a *spatial* distribution of neutrophil numbers within the section from the surface of the wound deeper into the dermis can be observed. An indication of this can be seen in the raw data in Appendix 10.5 and 10.6.

The distribution of neutrophil numbers between sections taken at the time of operation, and later at time 48 hours in both control and TNP-treated donor site wound biopsy sections, are illustrated in Figure 45. The graph shows that the distribution of neutrophils within the section is dependant on the distance from the wound surface, with the majority of neutrophils within the first 250µm of dermis with very few cell counts beneath 1000µm.

Furthermore, gross comparisons between the mean distribution of neutrophil cell counts from the t48 control wounds (n=6) with the mean distribution of neutrophil cell counts with their respective TNP-treated sites (n=6), demonstrates that there are fewer neutrophils in the TNP-treated groups at each 250µm incremental depth up to 1000µm. The numbers of neutrophils are reduced the deeper from the wound surface.

The distribution of neutrophils between control and TNP-treated deep partial thickness burn biopsy sections (Figure 46) and superficial partial thickness burn biopsy sections (Figure 47) are also illustrated below.

In a similar fashion to the donor site biopsy sections, the distribution of neutrophils in burn biopsy sections relate to the depth of the burn wound. Again, the greatest number of cells can be seen within the first 250µm of the wound in both partial thickness burn types. The difference between the mean control and TNP-treated neutrophil numbers at each incremental depth is less clear and no differences can be noted statistically. Nonetheless, there appears to be fewer neutrophils in the TNP-treated sections than controls.

Figure 45 The effects of TNP therapy on the spatial distribution of neutrophils within biopsy sections of donor site-injured dermis (n=6)

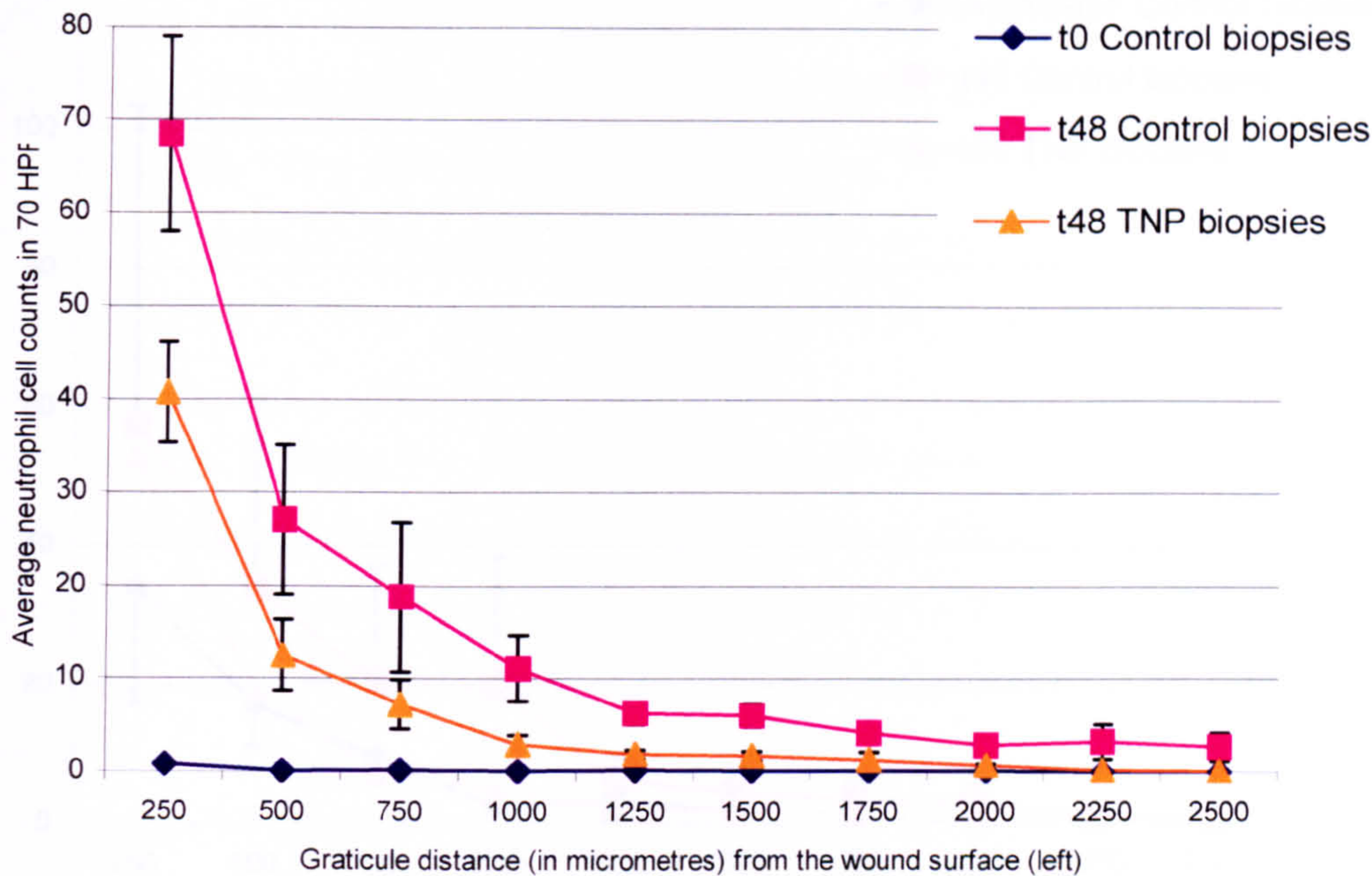


Figure 46 The effects of TNP therapy on the spatial distribution of neutrophils within sections of deep partial thickness burn dermis (n=3)

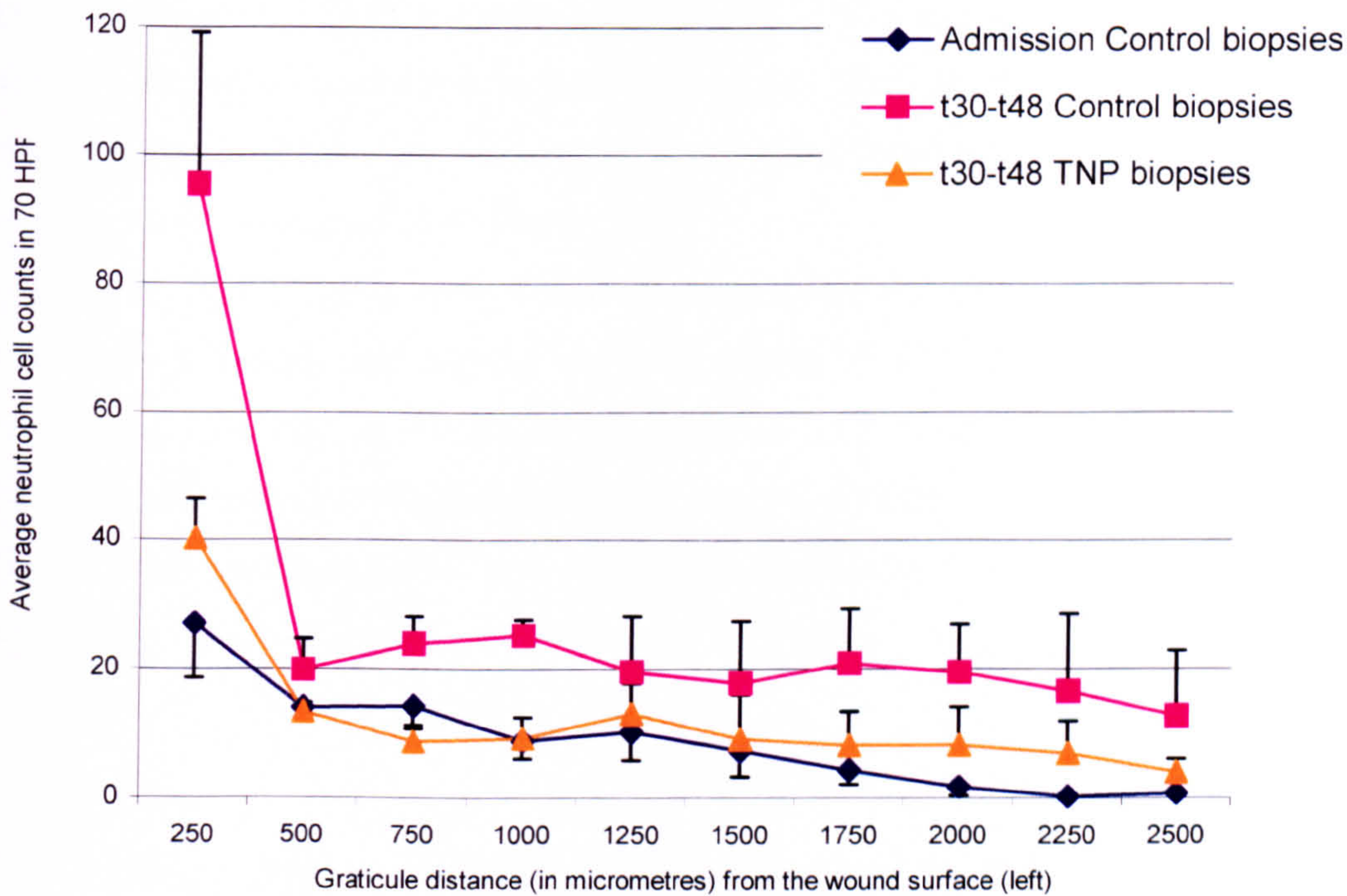
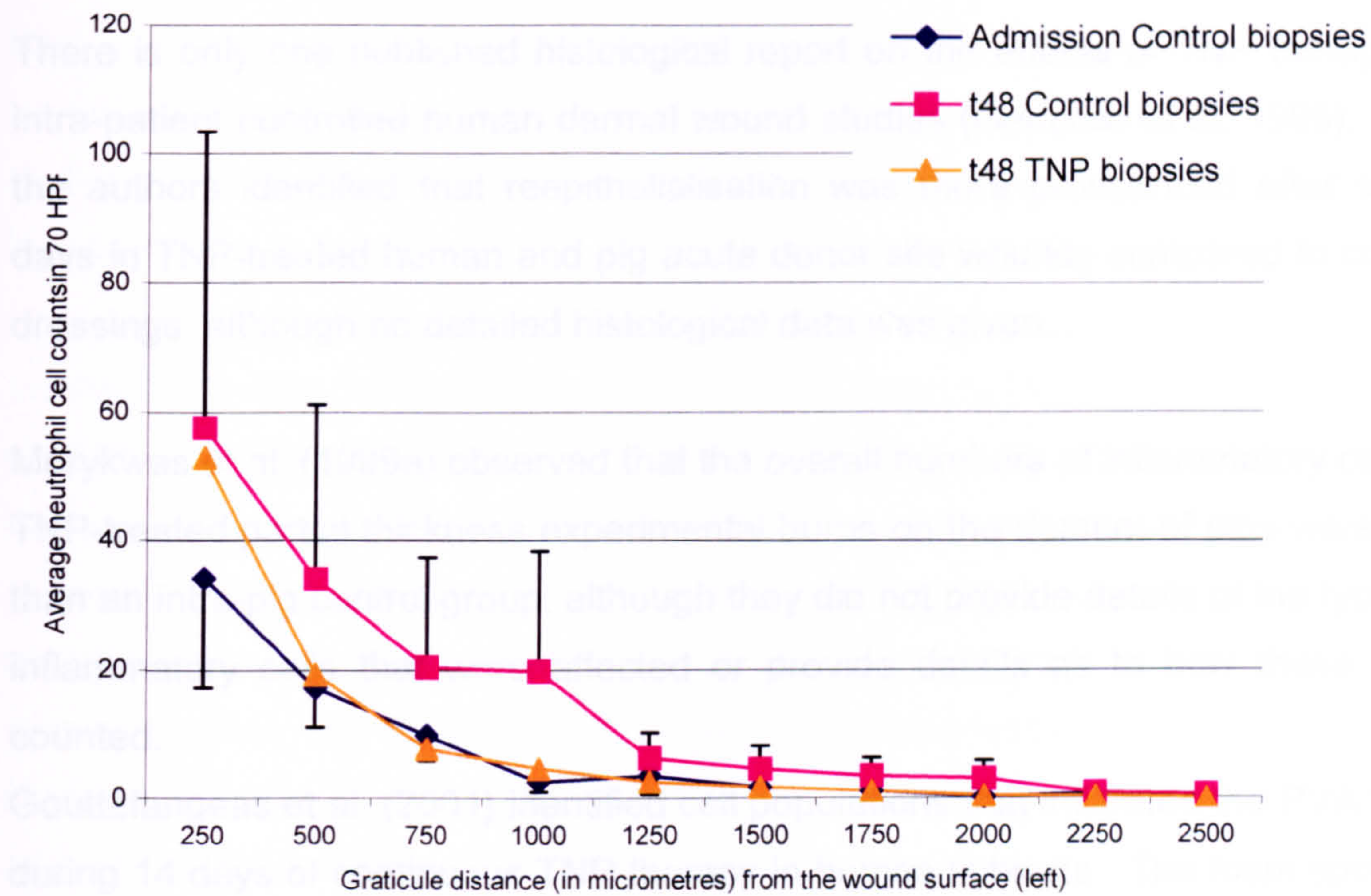


Figure 47 The effects of TNP therapy on the spatial distribution of neutrophils within sections of superficial partial thickness burn dermis (n=3)



There is only one histological report of a human burn wound treated with TNP. Ford et al (2002) have also shown that patients with TNP-treated wounds showed reductions in the level of bacterial species over time. In addition, plates collected at 100 micrometres from the wound surface in the first 24 hours of treatment with TNP showed a reduction in the number of bacteria. This suggests that TNP therapy may be acting directly on the bacteria from the wound bed (Ford et al, 2002). Mayhew et al (1997) have also shown that patients with TNP-treated wounds showed reductions in the level of bacterial species over time. In addition, plates collected at 100 micrometres from the wound surface showed a reduction in the number of bacteria. This suggests that TNP therapy may be acting directly on the bacteria from the wound bed (Ford et al, 2002). Ford et al (2002) have also shown in a randomized controlled trial of 22 patients with chronic ulcers undergoing either 4 weeks of TNP therapy versus control that the number of neutrophils decreased in the TNP group and increased in the control group (Ford et al, 2002). These findings support the view that TNP modulates cells and their number within wounds.

5.4.1 TNP modulates neutrophil cell count and their distribution

This study has demonstrated that TNP therapy is capable of reducing the number of neutrophils within the dermis as shown in both the donor and patient biopsies.

5.4 Discussion

There is only one published histological report on the effects of TNP therapy on intra-patient controlled human dermal wound studies (Genecov et al. 1998). In it, the authors identified that reepithelialisation was more pronounced after seven days in TNP-treated human and pig acute donor site wounds compared to control dressings, although no detailed histological data was given.

Morykwas et al. (1999a) observed that the overall numbers of inflammatory cells in TNP-treated partial thickness experimental burns on the dorsum of pigs were less than an intra-pig control group, although they did not provide details of the types of inflammatory cells that were affected or provide details as to how these were counted.

Gouttefangeas et al. (2001) identified cell populations that infiltrated the PVA foam during 14 days of continuous TNP therapy in human subjects. The foam sponges were fixed and sectioned to reveal that the dominant cell type seen within the foam were neutrophils. They observed a gradual decrease in cell distribution from the surface to the inner structure of the foam. This suggests that TNP therapy sucks cells up directly into the foam from the wound bed (Gouttefangeas et al., 2001).

Morykwas et al. (1997) have also shown that biopsies from TNP-treated pig wounds showed reductions in the level of bacterial species grown on blood agar plates compared to within-pig controls, suggesting that the bacteria might have been actively removed from the wound.

Ford et al. (2002) have also shown in a randomised controlled trial of 22 patients with chronic ulcers undergoing either 6 weeks of TNP therapy versus wound gel dressings, that the numbers of neutrophils decreased in the TNP group and increased in the wound gel dressings group (Ford et al., 2002).

These findings support the view that TNP modulates cells and their number within wounds.

5.4.1 TNP modulates neutrophil cell counts and their distribution

This study has demonstrated that TNP therapy is capable of reducing neutrophil numbers within the dermis, as shown in both the donor site and partial thickness

burn wound biopsy sections compared to controls, and that the distribution of NP57 positive-stained cells within the injured dermis of donor site and partial thickness burn wounds is also affected by TNP therapy when comparing the numbers of cells at the same distance from the surface of the wound. These findings are consistent with the effects of TNP on cells within wounds as described by others.

Interestingly, donor site wounds ID 2 and ID 4 were taken from patients 19 days post 4% full-thickness burn wounds and 11 days post 13% mixed partial thickness burn wounds, respectively. All the other donor site wounds were taken from patients requiring grafting for non-acute inflammatory conditions. Table 14 showed that average neutrophil cell counts for both the control and TNP-treated biopsy sections for donor sites ID 2 and ID 4 were less than those from other donor site biopsy sections. These differences might be accounted for, in part, by the relative systemic neutropenia seen in some burn patients although this cannot be verified. Unfortunately, the numbers of patients in this study are very few.

Although an objective assessment of neutrophil degranulation was not made in this study, there were no obvious (subjective) differences observed in degranulation between TNP-treated and control donor site and partial thickness burn wound biopsy sections, or between superficial and deep partial thickness burn biopsy sections.

This study does not provide any direct evidence to demonstrate how or why there are fewer neutrophils in the TNP-treated biopsies, although the following hypotheses might be proposed:

1. The action of subatmospheric pressure on the wound surface might remove neutrophils indirectly by sucking them up in wound fluid.
2. TNP might exert a direct effect on cell migration through effects of mechanical shear stress.

3. There may be few cells in the first instance since the effects of irrigating and applying suction to the wound may wash the wound surface compared to non-irrigated Kaltostat dressing (control) sites.
4. During the removal of the suction device, the top layer of fibrin exudate-containing cells may have been removed along with the foam dressing, thereby reducing the uppermost neutrophil-laden layer from the wound.

This thesis did not examine these four potential mechanisms directly. Despite this, there is good evidence to suggest that TNP might remove cells and cellular debris via its behaviour on the wound fluid. Firstly, cells and cellular debris have been observed in smears made from the pellets of centrifuged wound fluid samples taken from patient wounds undergoing TNP therapy without the use of an irrigant fluid vehicle which might 'wash' the wound surface of cells (Banwell, Personal communication). Secondly, it has been demonstrated that on/off TNP therapy is capable of modulating the removal of proteins directly within wound fluid as shown in Chapter 4, and therefore might modulate cell numbers in a similar fashion. Unfortunately, this thesis did not attempt to identify cells or their numbers in the wound fluids from those samples taken during the on- and off- phases of suction, which might have answered this question. Thirdly, the effects of TNP therapy appeared more marked on the neutrophil distribution in donor site wound than partial thickness burn wound biopsy specimens. The donor site wound creates a blood-exposed exudate with potentially direct access to the neutrophils within the intravascular system since the donor site wound exposes the ends of dermal capillaries to the TNP device. Meanwhile, the partial thickness burn wound is a 'plasma-free' wound because the surface of the wound is burn-sealed without direct contact to the dermal capillaries. This may reduce the capacity of the subatmospheric pressure to pull neutrophils (accompanying the fluid) out of the wound directly.

The implication that TNP therapy, by reducing neutrophil cell counts within dermal wounds, might be beneficial to wound healing remains highly speculative, and is not suggested from this work. This thesis did not examine whether TNP therapy actually improves the clinical outcome in donor site wound or partial thickness

burn wound healing, despite some evidence that this might be do so (Genecov et al. 1998; Morykwas et al. 1999) and made no attempt in defining the role of the neutrophil in this system.

Great care is taken to ensure that TNP-treated and control groups are as identical as possible. This is easier to establish with the donor site wound in which a uniform, mechanical injury was created on the skin using an electrical dermatome. Although it was essential to choose identical looking partial thickness burn wounds for both the TNP and control sites, the decision was based solely on clinical inspection, pinprick testing and capillary return. Objective, independent confirmation of the depth (extent) of the wound was not obtained for no satisfactory, accurate technique was available.

Few references in the literature describe techniques for cell counting within sections of human skin. The main problem is that the distribution of the cells within the dermis is *not* random, as can be seen in these sections and which are clearly illustrated in Figures 45 to 47. Inflammatory cells are often localised in and around blood vessels. Sections of injured dermis with greater vessel density might increase the neutrophil cell counts within that section. Control biopsies are taken about 6-10 cm away from TNP biopsies within the same patient and it cannot be guaranteed that the same vessel distribution is present. Despite this, when the mean cell counts are compared between control and TNP biopsy sections using a grid which covers the whole section, any differences in perivascular neutrophil numbers is not observed. This study has demonstrated that neutrophils are most prevalent near the wound surface, therefore taking random HPF counts of a section of a biopsy wound would yield marked differences in cell counts depending on where in the dermal section the counts are made. Hence, a 7x10 fixed grid map may be a more accurate method of counting cells within dermal sections. This study has questioned the reliability of 'random' cell counting under the microscope, as an accurate means of determining cell populations within dermal biopsy sections.

If the results of this study are true, then the effects of TNP therapy on neutrophil numbers might be highly significant to the concept of burn depth and its

progression. For example, Tyler (1998) has observed that the extravasating neutrophil counts in partial thickness human thermal burns correlated directly with burn depth. Bucky et al. (1994), Choi et al. (1995) and Nwariaku et al. (1996) have shown experimentally that by blocking extravasating neutrophils from within burn wounds improved microvascular blood flow, and secondary progressive dermal necrosis. In a similar fashion, TNP therapy on burn wounds might also reduce the effects of neutrophil-mediated tissue injury by both enhancing blood flow and also by reducing the neutrophil numbers in the burn wound. This histological study did not examine the direct effect of TNP therapy on burn depth progression between TNP-treated versus control wounds. This would represent interesting future work and might support the experimental findings of Morykwas et al. (1999a) in pig studies, suggesting that TNP therapy might prevent progressive partial thickness burn injury.

There are a number of criticisms of this study mentioned below.

Firstly, donor site sections stained heavily for neutrophils within the fibrin clot on the surface of each wound. It was important to delineate the surface of the wound carefully in each case, and exclude the wound surface and fibrin clot during neutrophil cell counting to be certain not to include these cells in the overall cell counts. It was important to establish the wound surface accurately so that comparisons could be made between TNP-treated and Kaltostat-treated (control) wounds could be made. This was also important when comparing treated and control burn wounds.

The optimum dressing used on the control wound would ideally have been the TNP device alone without suction, instead of the standard Kaltostat dressings used in the treatment of donor site wounds and partial thickness burns, as this is the biggest drawback to this study design. Indeed, one reason for the reduction in neutrophil cell counts observed in the TNP-treated sections may be due to the fact that after 48 hours of TNP therapy, the semi-adherent foam dressing is gently peeled off the wound surface. This might have created a mechanical debridement of the top layer of the wound and reduced the number of neutrophils on or near the surface. Although this might explain the reported reduction in neutrophil counts in TNP-treated biopsy sections, care was taken to count only neutrophils *within* the dermis and not those cells on the surface.

However, in support of this work, there was no evidence of dermal tissue sticking to the foam itself when it was removed and the depths of the dermis in each biopsy section between paired TNP-treated and control sections did not differ, suggesting that peeling off the TNP dressing did not remove layers of upper dermis. However, no accurate measurements of the depth of the dermis was made. Future studies using this TNP device might best be achieved by placing a layer of non-adherent dressing (such as Mepital™) between the wound and foam to prevent any foam from adhering to the wound surface. In this fashion, 'foam-stripping' of the wound surface might be prevented, and a more accurate method of histological assessment may be made. Additionally, the control group should ideally be the foam dressing alone, although this might be difficult for the ethics committee to accept.

Secondly, TNP therapy was used in conjunction with the irrigation device and not with the standard TNP device. It might be useful to repeat the study without the irrigation effect on the wound surface and with continuous (rather than intermittent) suction since this may have an effect on the neutrophil numbers.

Thirdly, it must also be recognised that the immunohistochemical staining for Neutrophil Elastase using the monoclonal antibody, NP57, might also stain for other Neutrophil Elastase-expressing cells. Indeed, a subpopulation of macrophages are also known to express Neutrophil Elastase on their cell surface and represent up to 6.2% of Neutrophil Elastase content (Campbell et al, 1989). However, Herrick et al. (1997) staining for Neutrophil Elastase in acute and chronic tissue biopsies, confirmed that most of their cells expressing elastase, using NP57, were found to be granulocytes by double localization with the marker CD15. However, they reported some elastase expressing cells, mainly in the deep dermis, that were not CD15-positive, which instead displayed a fibroblast-like phenotype. Despite these potential inconsistencies, the same methodological staining procedure was used in this study, in both TNP-treated and control sections within the same patient and staining performed in the same batch, so it might be assumed that any differences in neutrophil and macrophage staining for Neutrophil Elastase would be relative to both groups. No attempt was made to carry out dual staining procedures in this case.

5.4.2 Advantages in using the TNP device in wound healing studies

The differences between neutrophil cell counts between wounds of the same type vary significantly. This finding supports the need for robust intra-patient control groups, which can be provided by using the selective TNP device on the same wound type with closely matched wound controls as described above. The error associated with the use of historical or inter-patient control groups becomes more evident. Although biopsy collection times between burn patients differed according to their clinical situation, the exact timing was less crucial if intra-patient matched controls could be obtained.

5.5 Conclusion

Selective application of TNP using the irrigation device described in this thesis has provided the opportunity to directly compare the neutrophil cell counts in sections of biopsies taken from TNP-treated and adjacent control wounds in donor site and partial thickness burn wounds.

Neutrophil cell counts from within the dermis of six paired donor site wounds and six paired partial thickness burn wounds has shown that TNP-treated areas demonstrate reduced neutrophil numbers. The significance of this finding to wound healing is not clear. It is proposed that the effects of subatmospheric pressure on a wound surface promote the removal of cells by its effect on wound fluid, although a direct mechanical effect of TNP on cell motility and adhesion cannot be ruled out.

The distribution of neutrophils up to 1000µm within the depth of the dermis also appears to be reduced by the effects of TNP. This finding highlights the need to define the exact location of neutrophils within the dermis, since random cell counts within the dermis has been shown not to be an accurate method of cell counting.

6 The influence of Topical Negative Pressure therapy on the temporal analysis of Neutrophil Elastase activity and α_1 Protease Inhibitor in wound fluid of human donor site and partial-thickness burn wounds

6.1 Introduction

The potent serine protease, neutrophil elastase (NE) is released from neutrophils during inflammation. It has broad substrate specificity and when released in excessive amounts, is capable of significant pathological tissue damage. Indirect evidence suggests that NE might contribute to impaired wound healing by degrading provisional extracellular matrix proteins. There is good evidence that NE activity is increased in some tissues of poor-to-heal cutaneous wounds and in wound fluids of both chronic wounds and burn wounds.

α_1 -protease inhibitor (α_1 -PI) is the most important endogenous inhibitor to NE activity in vivo. An imbalance between the relative levels of NE and plasma-derived α_1 -PI may play an important part in the failure or delay of some wounds to heal.

TNP therapy is widely used in the treatment of acute and chronic wounds, although the exact mechanisms of action are not understood. One such mechanism may involve a direct ability of TNP to modulate the constituents of the wound fluid (including tissue damaging proteases) that might be sucked out of cutaneous wounds.

Since on/off TNP therapy is capable of modulating the total protein concentration of wound fluids as demonstrated in Chapter 4, and that neutrophil cell counts are reduced in wounds under the influence of TNP as shown in Chapter 5, this study was designed with the following aims:

1. To determine the relative levels of NE activity within the wound fluid samples (from donor site and partial thickness burn wounds) during 'on/off' TNP therapy

2. To determine the relative concentration of α_1 -PI in the same samples
3. To identify whether 'on/off' suction TNP therapy is capable of modulating the NE/ α_1 -PI balance

6.2 Materials and Methods

6.2.1 Wound fluid Neutrophil Elastase activity assay

The same set of wound fluid samples taken from patients with donor site wounds (Table 9 on page 114), and burn wounds (Table 10 on page 115) were used.

Two different NE immunochemical assays were performed on both sets of wound fluid samples in this study. Both activity assay methods employed in this study are detailed below.

6.2.2 Colourimetric NE assay of fluid samples

Principles:

An immunochemical spectrophotometric assay takes advantage of the ability of a coloured solution to absorb light of a specific wavelength. The absorption of light is related directly to the concentration of the coloured substances in the solution (Beer-Lambert Law). In the following assay, a commercially prepared substrate combines irreversibly to human NE in both known and unknown fluid samples on a stoichiometric 1:1 ratio, to liberate a yellow colour change in the substrate, which is measured colourimetrically.

Reagents:

- A. 100µg human neutrophil elastase (Calbiochem, UK.) was diluted in 1ml ddH₂O to make up a stock solution (100µg/ml). Following reconstitution, the stock solution was stored at -20°C in aliquots of 100µl until required.
- B. 10mg chromogenic elastase substrate (Calbiochem, UK.) was stored at -20°C until required. This product is a very specific, synthetic, nitroanilide (NA) substrate (Methoxysuccinyl-Ala-Ala-Pro-Val-pNA) for NE.
- C. 0.1M HEPES buffer (pH 7.4) was freshly prepared as described in the Appendix (section 10.3 on page 200).

The colourimetric assay technique (after Nakajima et al., 1979) is described below:

Using the total protein data from Chapter 4, a small volume of each wound fluid sample (<70µl) containing an equivalent amount of protein was pipetted into a 96-microwell plate in triplicate. HEPES buffer (pH 7.4) was added to make up to 100µl.

50µl human neutrophil elastase (Calbiochem, UK.) standards ranging from 5 – 0.01µg/ml diluted in 0.1M HEPES buffer (pH 7.4) were also pipetted into the 96 microwell plate in triplicate. HEPES buffer (pH 7.4) was also added to make up to 100µl. HEPES buffer test blanks (100µl) were also added in triplicate.

100µl of 6mM elastase substrate, MeOSuc-Ala-Ala-Pro-Val-pNA (dissolved in 10% dimethyl sulfoxide, DMSO) diluted in 0.1M HEPES buffer (pH 7.4) was added to each well plate. The plate was incubated for 1 hour at 37°C and substrate degradation was determined by measuring the absorbance spectrophotometrically at 405nm.

A standard curve for degradation was prepared using the known set of standard NE concentrations and plotted to determine an equation from which the unknown wound fluid samples could be compared. The results were expressed as units/ml of NE activity/100µg Total Protein ($\times 10^{-4}$) and displayed graphically using Excel 2000 software.

6.2.3 Fluorometric NE assay of fluid samples

Principles:

A photometric assay using the same synthetic NE substrate (MeOSuc-Ala-Ala-Pro-Val-pNA) can be used when coupled to a fluorescent 7-amino-4-methylcoumarin (7-AMC) compound. The (almost) nonfluorescent substrate is hydrolysed by NE to liberate the intensely fluorescent 7-AMC, which is quantified fluorometrically (Barrett et al., 1981). The intensity of fluorescence is directly proportional to the concentration of the reaction between substrate and NE. A molecule at the ground state energy level is excited by light absorption to a higher

excited energy level. As the molecule returns to the more stable ground state level, light is emitted. The instrument for measuring fluorescence differs from that of spectrophotometers in that a fluorometer contains two monochromators, one to regulate the wavelength of light striking the sample and one to isolate the desired wavelength of light emitted from the sample following excitation. The sensitivity of the fluorometric assay may be 10^3 times that of absorption spectrophotometry (Nguyen et al., 1996).

Reagents:

- A. MeOSuc-Ala-Ala-Pro-Val-pNA(-7-AMC) (Bachem, UK) is dissolved in DMSO as a 1mM solution and stored at 4°C
- B. 0.1% Triton X-100
- C. 0.2M Tris-HCl buffer is prepared at pH 8.5
- D. Soybean-trypsin inhibitor (Sigma Chemical, UK.) in ddH₂O (100µg/ml) is prepared as the stopping solution

The fluorometric assay technique (after Barrett et al., 1981) is described below:

500µl of sample fluid, diluted (1:10) in 0.1% Triton X-100, was prepared in triplicate in labelled glass tubes. 500µl of a 10nM NE standard solution, diluted in Triton X-100, was also prepared in triplicate. Blanks were prepared with 500µl of Triton X-100 in triplicate. Each set of patient samples were analysed in the same batch run.

250µl of Tris-HCl buffer was mixed with each sample solution, NE standard and blanks and the tubes warmed in a water bath for 5 minutes at 40°C.

250µl of the MeOSuc-Ala-Ala-Pro-Val-pNA(-7-AMC) substrate stock solution (1mM), diluted to 20µM in ddH₂O, was added to each tube in the water bath to start the reaction. The reaction was stopped at 10 minutes by the addition of 1ml of soybean inhibitor (100µg).

Each sample solution was transferred to a glass cuvet and placed in a fluorometer (Perkins Elmer Model LS0B, Norwalk, CT, USA). The sample was excited with

light at 350nm and the release of 7-AMC was measured at 460nm. A fluorescence reading, taken over a 10 second integration time, was displayed on a Windows 95 computer screen and recorded for each of the samples.

The following equation was used to calculate the NE activity ($\mu\text{mol}/\text{min}/\text{litre}$ or arbitrary units of activity/litre) of each unknown sample in triplicate:

$$\frac{(\text{reading of sample} - \text{reading of substrate blank}) \times (\text{dilution factor}) \times (\text{vol. of enzyme in mls})}{(\text{average of standards}) \times (\text{number of minutes of the reaction})}$$

where:

reading of substrate blank was the average of three blank readings

the dilution factor = 10 (1:10)

vol. of enzyme in mls = 0.5

number of minutes for the reaction = 10

NE activity in units/litre were plotted for each sample over time using Microsoft Excel 2000 software.

Using this method, the generation of a standard curve for a series of known NE solutions is not required, reducing the amount of NE and its fluorogenic substrate required for the assay.

Coefficient of variation: intra assay determination 1.3%
 inter assay determination 1.92%

Each set of 20 wound fluid samples per donor site patient and each set of partial thickness burn wound fluid samples (between 8 and 16 samples) were measured in triplicate, separately in batches, in both a 96-well plate (colourimetric assay) or in glass tubes (fluorometric assay).

The emphasis of these experiments was to identify differences in neutrophil elastase in within-patient samples during on/off suction.

6.2.4 Nephelometry measurements of α_1 -PI in wound fluid samples

Principles:

Nephelometry is the measurement of antigen-antibody complexes formed in enzyme immunoassays. The occurrence of immune complex formation is related to the amount of light scattering and is the basis of antigen quantification (Kasahara and Nakamura, 1996). The instrument measures scattered light during the formation rate of insoluble immunoprecipitation products resulting from a specific antigen combining with a specific antibody. Fully automated testing of many proteins can be performed using this technique.

The method for measuring α_1 -PI was established using rate nephelometry on a Beckman Image Array (Beckman, Coulter Instruments, UK). The identical wound fluid samples were analysed. This work was kindly performed courtesy of the staff at the Protein Reference and Immunopathology Unit, St. George's Hospital. The results were displayed as g/l and represent total α_1 -PI concentration.

This automated method was standardised with processed human serum (Beckman, UK). A calibrant was sold and cross-calibrated against the International Reference Preparation for plasma proteins, CRM 470 (Whicher et al. 1994).

This method of measurement is used in the Protein Reference Unit for the routine determination of serum albumin, immunoglobulins complement and other plasma proteins (including α_1 -PI). This method measures total α_1 -PI, including both bound and free anti-protease. For quality control purposes, Beckman quote within batch coefficient of variation of <5%, which has also been confirmed on informal instrument evaluation in the laboratory (Personal Communication, Dr. J. Sheldon). Only one measurement per sample was obtained, rather than in triplicate.

6.3 Results

Neutrophil elastase in the donor site samples was measured using the fluorometric activity assay and the results are reported in section 6.3.1 below. Neutrophil elastase in the burn wound fluid samples was measured using both the colourimetric activity assay and fluorometric activity assay, and the results reported in section 6.3.2 below.

6.3.1 Neutrophil elastase activity assay of donor site wound fluids

The results of the fluorometric activity assay for donor site wound fluids are displayed in Figure 48 below. The neutrophil elastase activity in each sample is modulated by 'on' and 'off' suction cycles. In the same way as total protein concentrations, the elastase level is greatest during the 'on' phase and drops during the 'off' phase ($P < 0.0035$). There appears to be wide variations in elastase activity between the six donor site wounds ($P = 0.0001$), but the variation within the 20 sample cases remained consistent.

The NE activity in wound fluid samples from ID3 and ID5 are greater than those of ID2, ID4, ID6a or ID6b ($P = 0.05$). These latter four donor site wound fluid samples were all obtained from patients that had been previously burnt (see Table 9 on page 114). ID6a and ID6b were donor site wounds taken from a man with 40% burns.

6.3.2 Neutrophil elastase activity assay of partial thickness burn wound fluids

The results of the colourimetric (Figure 49) and fluorometric (Figure 50) activity assays for each of the burn wound fluid samples are shown below.

The two assays yield different results with respect to the effects of 'on' and 'off' suction for identical samples (both measured in triplicate).

The colourimetric assay of the burn wound fluid show that on/off suction does not appear to modulate NE activity ($P=0.57$). Furthermore, the relative NE activity in each set of samples differ to each other, although there appears to be greater activity in the clinically deep partial thickness burns, ID A, ID Ba and ID Bb than the clinically superficial partial thickness burns, ID Ca and ID Cb ($P=0.0002$).

Meanwhile, the fluorometric assay of the burn wound fluid (Figure 50) show that on/off suction *does* appear to modulate NE activity ($P=0.0002$) with on = highest, and off = lowest. There appeared to be no difference in overall activity between the clinically deep and clinically superficial partial thickness burns ($P=0.46$).

6.3.3 Neutrophil Elastase activity between wound types

Since the same fluorometric assay was performed on the donor site and burn wound fluid samples, the neutrophil elastase results can be broadly compared. There is no evidence in this study that the activity within wound fluid samples is greater in the burns than donor sites ($P=0.9091$). The highest activity in the burn samples is 0.16 units/litre (ID Ba, t12 hours) and the lowest is 0.05 units/litre (ID Cb, t43 hours). The greatest activity in the donor site samples is 0.27 units/litre (ID 5, t26 hours) and the lowest is 0.009 units/litre (ID 6b, t36 hours).

Figure 48 Neutrophil elastase activity in the six donor site wound fluid samples (determined using the fluorometric activity assay)

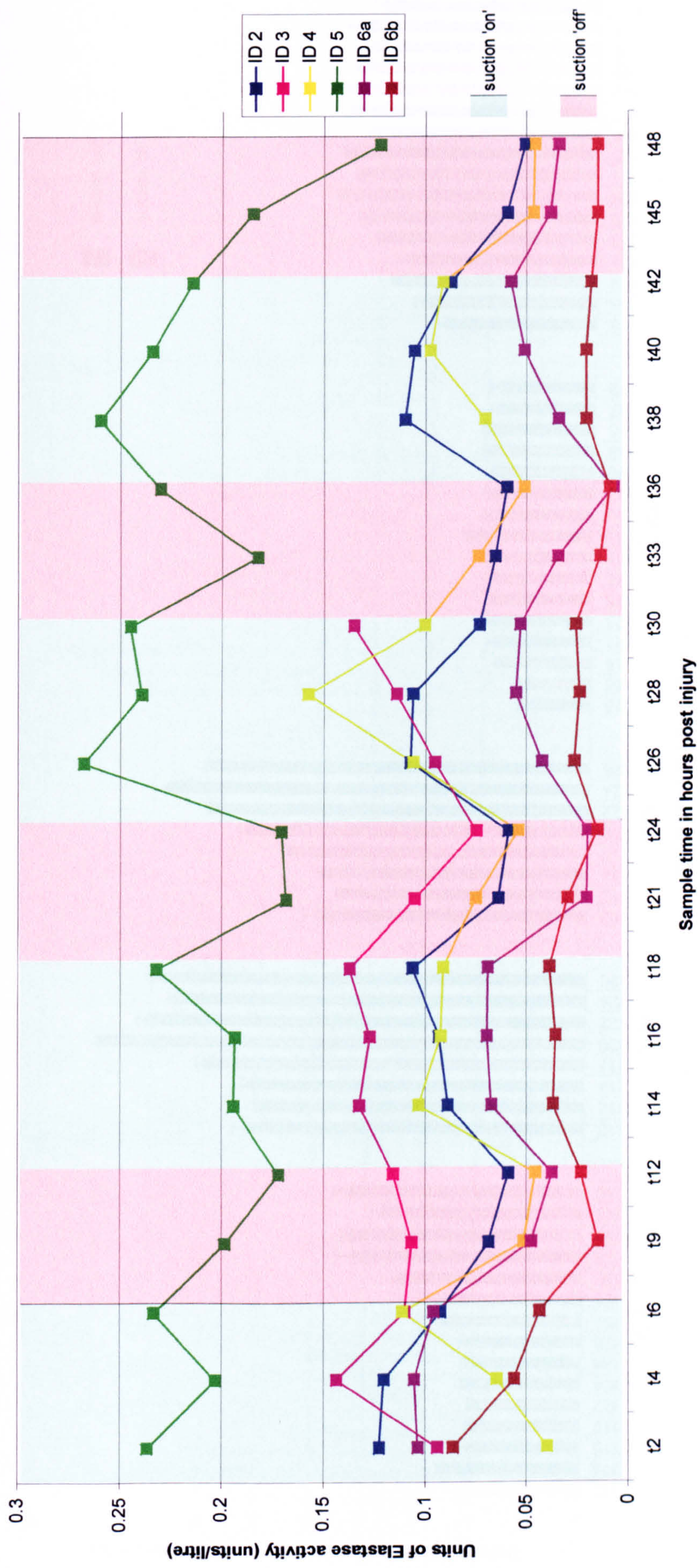


Figure 49 Neutrophil elastase activity in burn wound fluid samples (determined using the colourimetric activity assay)

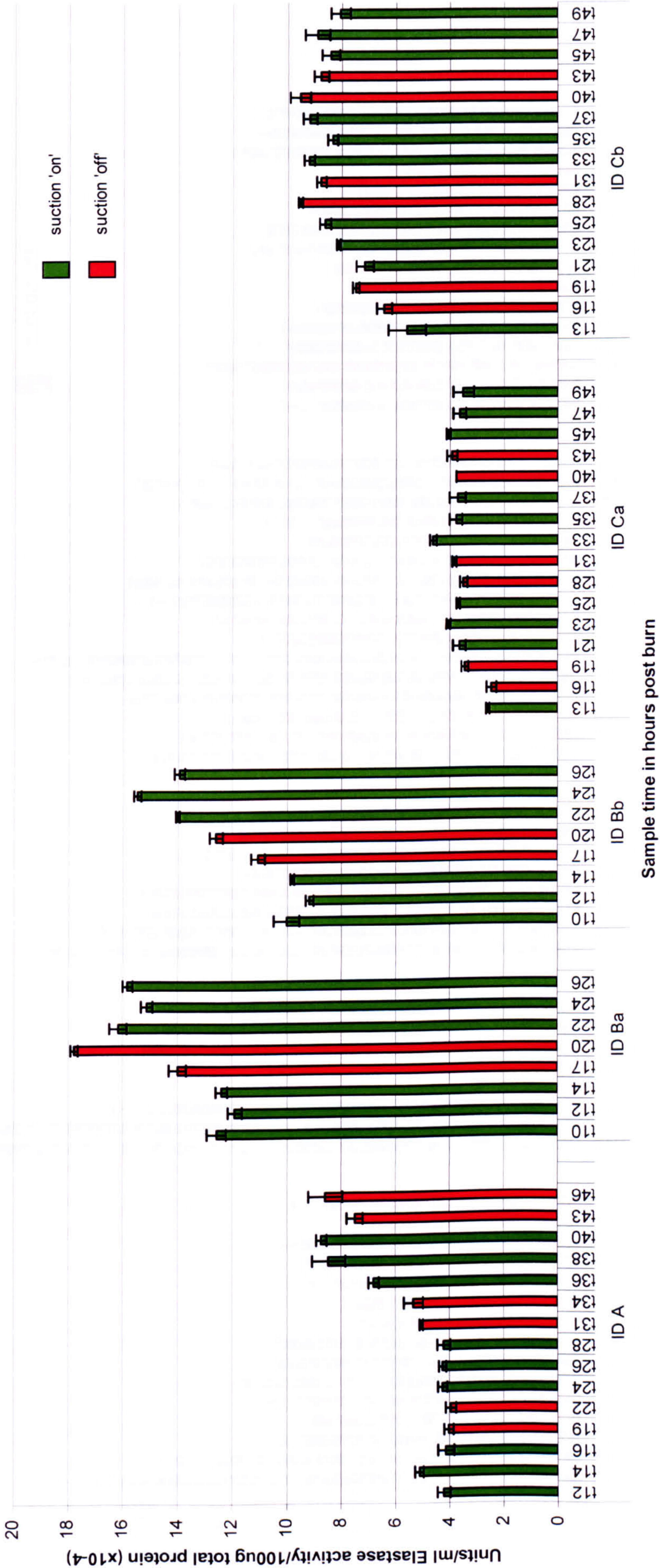
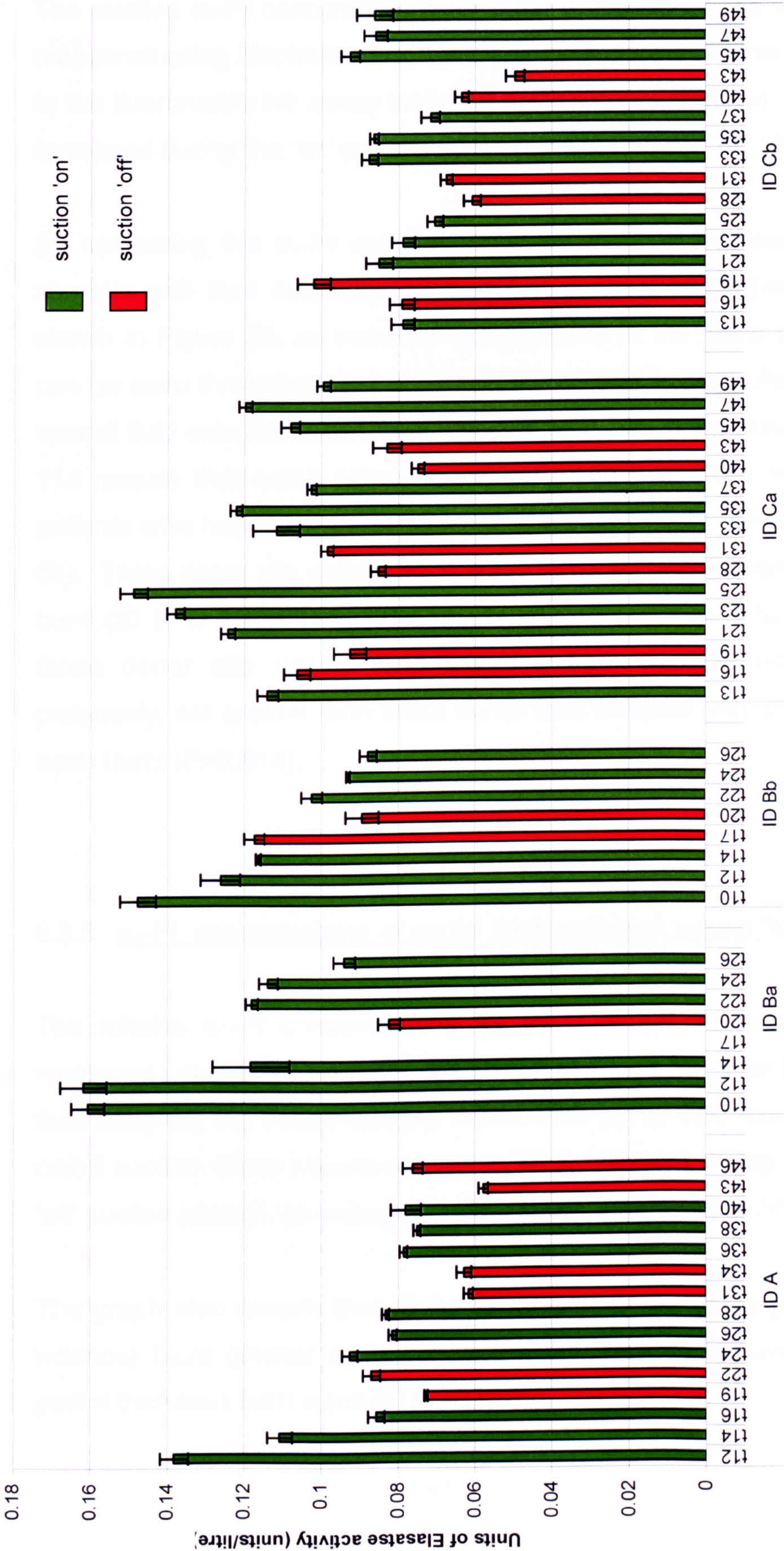


Figure 50 Neutrophil elastase activity in burn wound fluid samples (determined using the fluorometric activity assay)



6.3.4 α_1 -PI concentrations of donor site wound fluids

The relative α_1 -PI concentrations for each of the donor site wound fluid samples, measured using Nephelometry, are shown in Figure 51 below. In a similar fashion to the fluorometric NE assay and total protein concentrations, α_1 -PI concentrations increased during the 'on' phase and dropped during the 'off' phase.

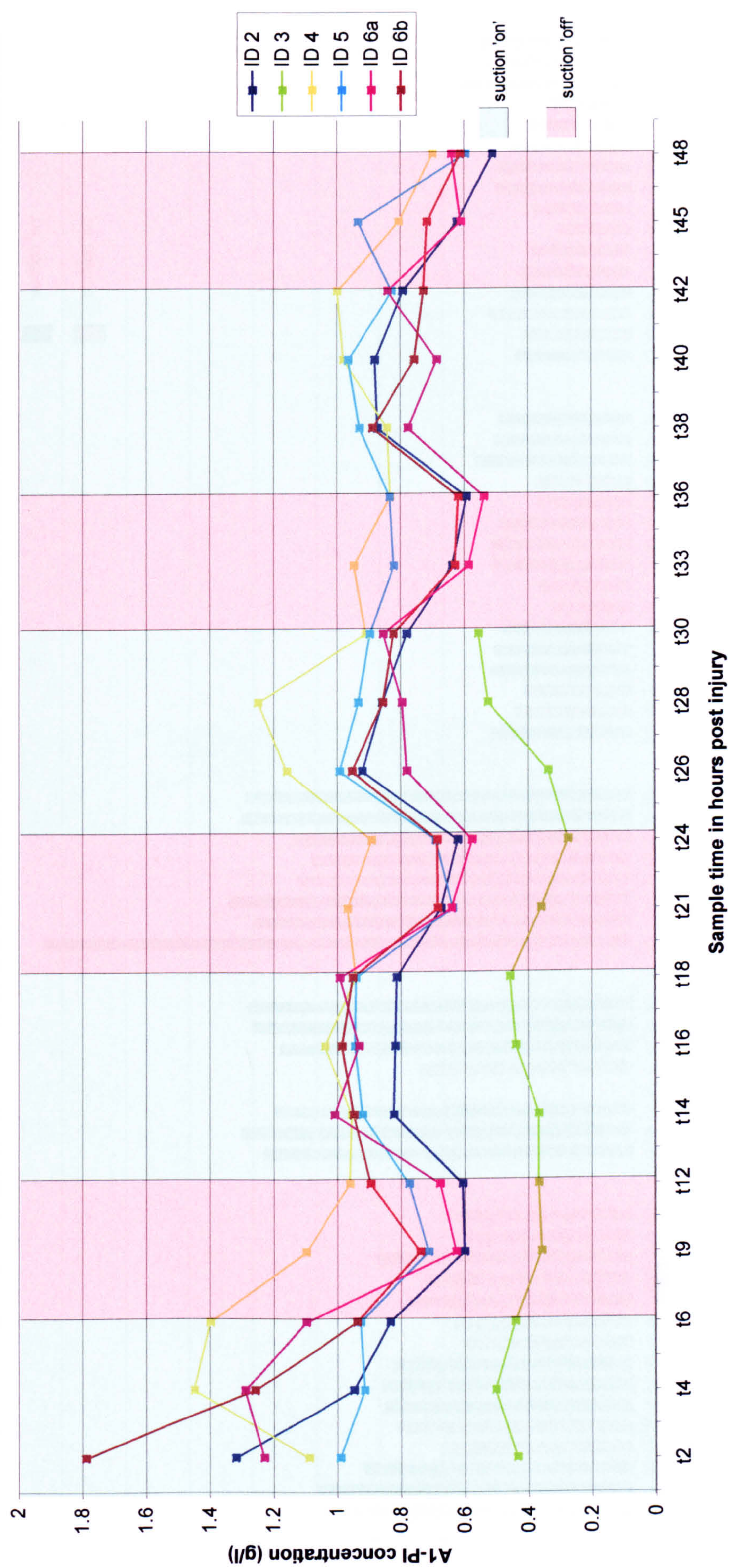
By comparing the α_1 -PI concentrations for each of the donor site wound fluid samples with their respective total protein concentrations (as a denominator), as shown in Figure 53, an increase in the nature of the acute phase protein, α_1 -PI, can be seen throughout the collection times, and in particular in those donor site wound fluid samples taken from patients who had been burnt. Table 9 on page 114 reveals that some wound fluids were obtained from donor site wounds of patients who had been burnt up to 19 days previously (ID 2, ID 4, ID 6a and ID 6b). Three donor site wound fluids were obtained from patients who had not been burnt (ID 1, ID 3 and ID 5). The results show that the α_1 -PI to total protein ratios in those donor site wound fluid samples from patients who had been burnt previously, are greater than those donor fluid samples from patients who have not been burnt ($P=0.014$).

6.3.5 α_1 -PI concentrations of partial thickness burn wound fluids

The relative α_1 -PI concentrations for each of the burn wound fluid samples, measured using Nephelometry, are shown in Figure 52. Like the donor site wound fluid samples, the concentrations within each set of fluid samples are affected by on/off suction. More plasma-derived α_1 -PI is obtained during the 'on' suction than 'off' suction phases, as indicated by the green and red bars ($P<0.0001$).

The graph also reveals that ID A, ID Ba and ID Bb (deep partial thickness burn wounds) have greater α_1 -PI concentrations than ID Ca and ID Cb (superficial partial thickness burn wounds) ($P=0.0392$).

Figure 51 α_1 -PI concentrations in the six donor site wound fluid samples (determined by Nephelometry)



PAGE

NUMBERING

AS ORIGINAL

Figure 52 α_1 -PI concentrations in the partial thickness burn wound fluid samples (determined by Nephelometry)

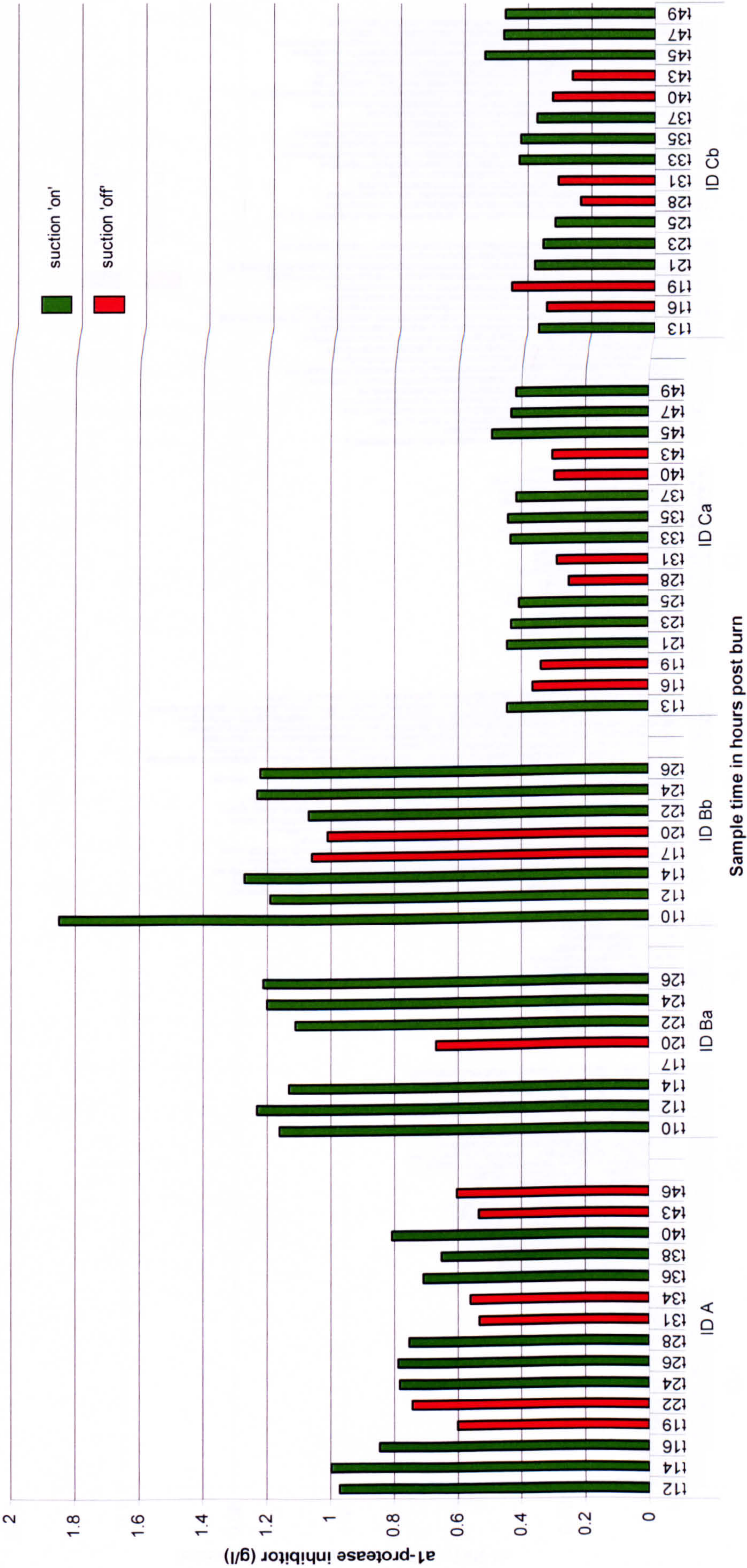
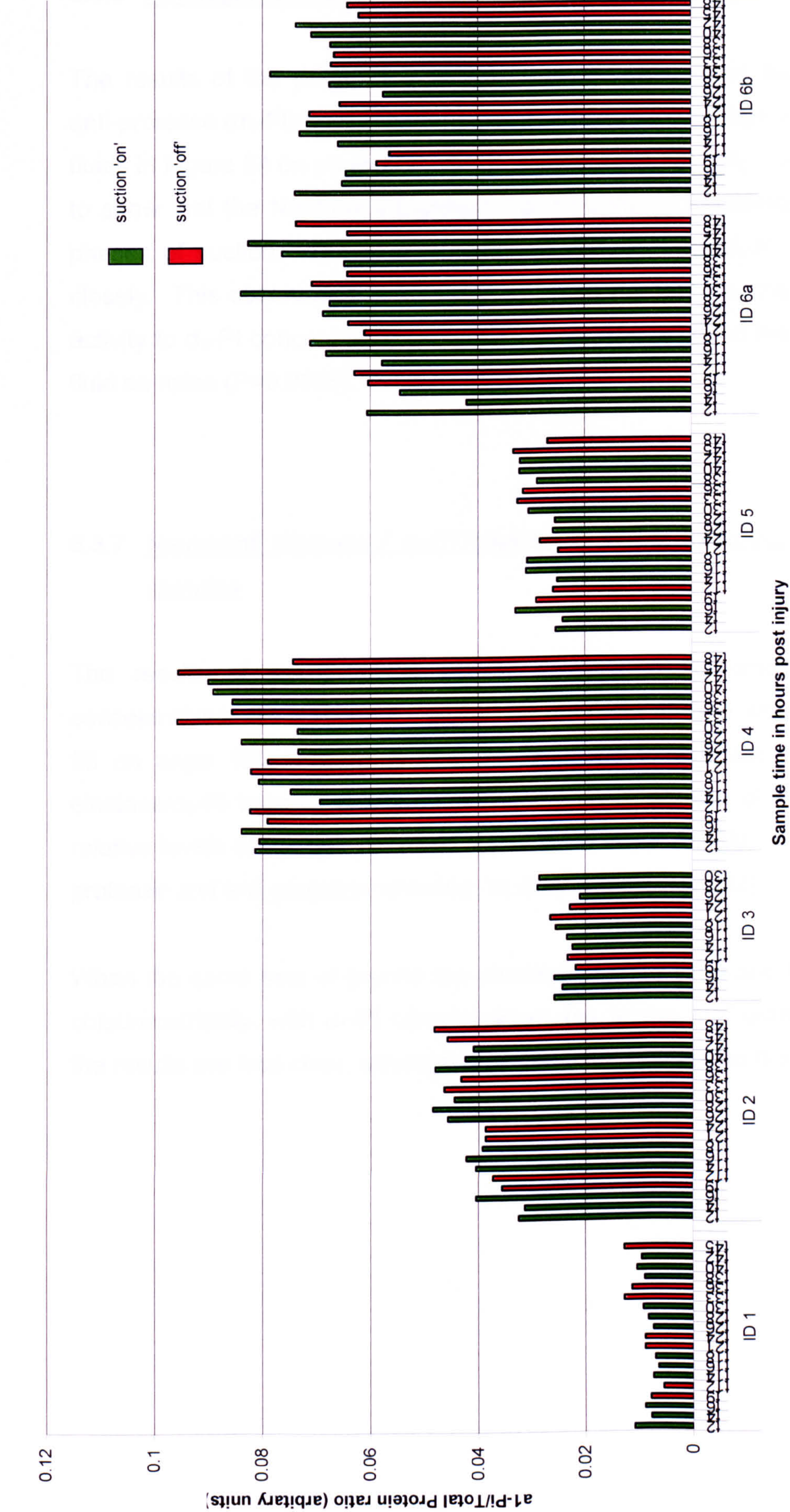


Figure 53 α_1 -PI against Total Protein for the donor site wound fluid samples. (Note that ID2, ID4, ID6a and ID6b are taken from burn patients)



6.3.6 Neutrophil Elastase / α_1 -PI balance in the donor site wound fluid samples

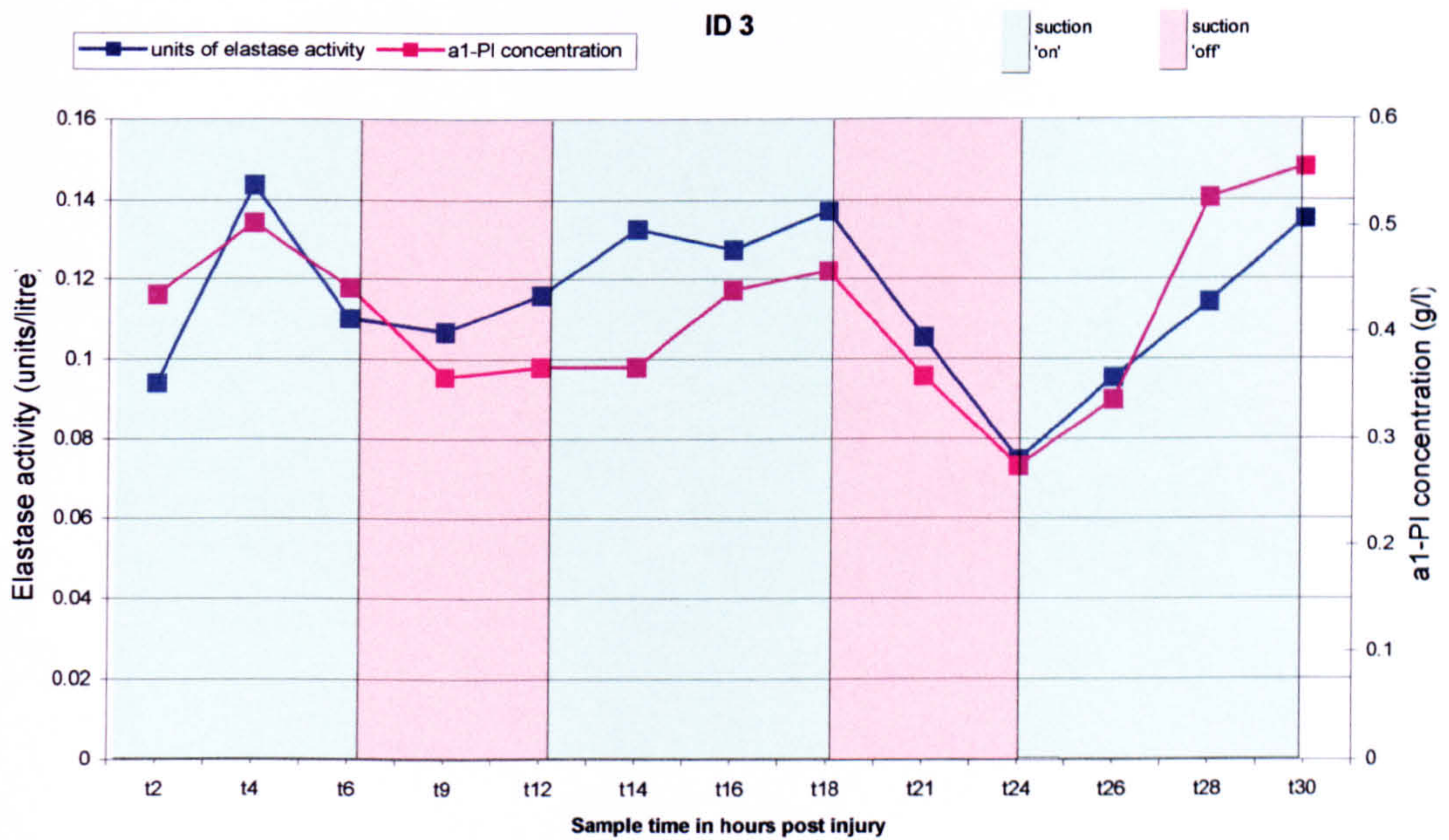
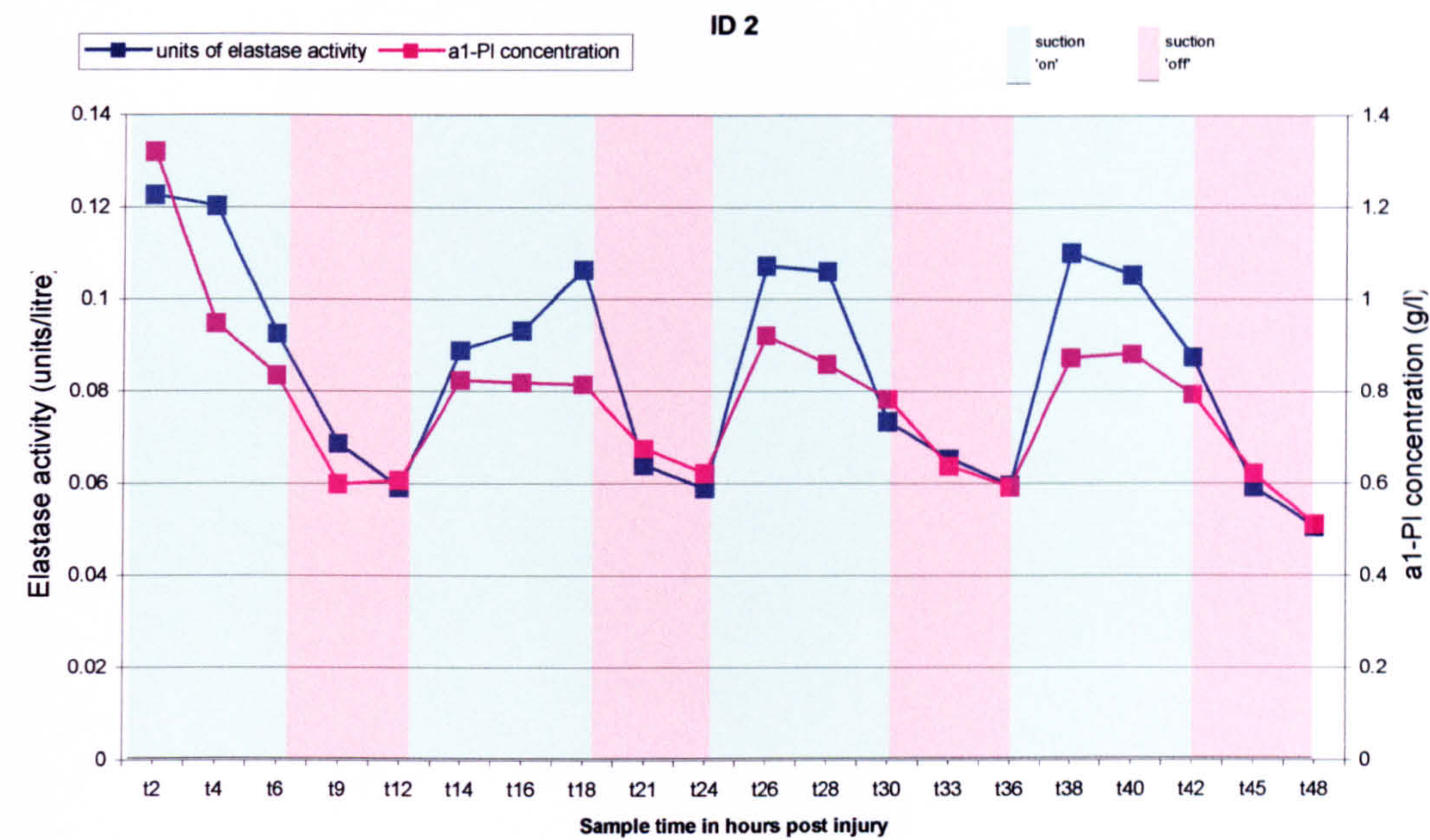
The results of the protease (elastase) activity, determined fluorometrically, and anti-protease (α_1 -PI) concentrations are displayed for each set of donor site wound fluids in Figure 54 on page 177. When combined in this way, there is no evidence to show that the Neutrophil Elastase / α_1 -PI balance is altered during the on/off phases of suction. The relative levels of elastase and α_1 -PI mirror each other closely. This observation can be supported by the fact that the ratios of both NE activity to α_1 -PI concentration demonstrate no differences in the donor site wound fluid samples ($P=0.2926$).

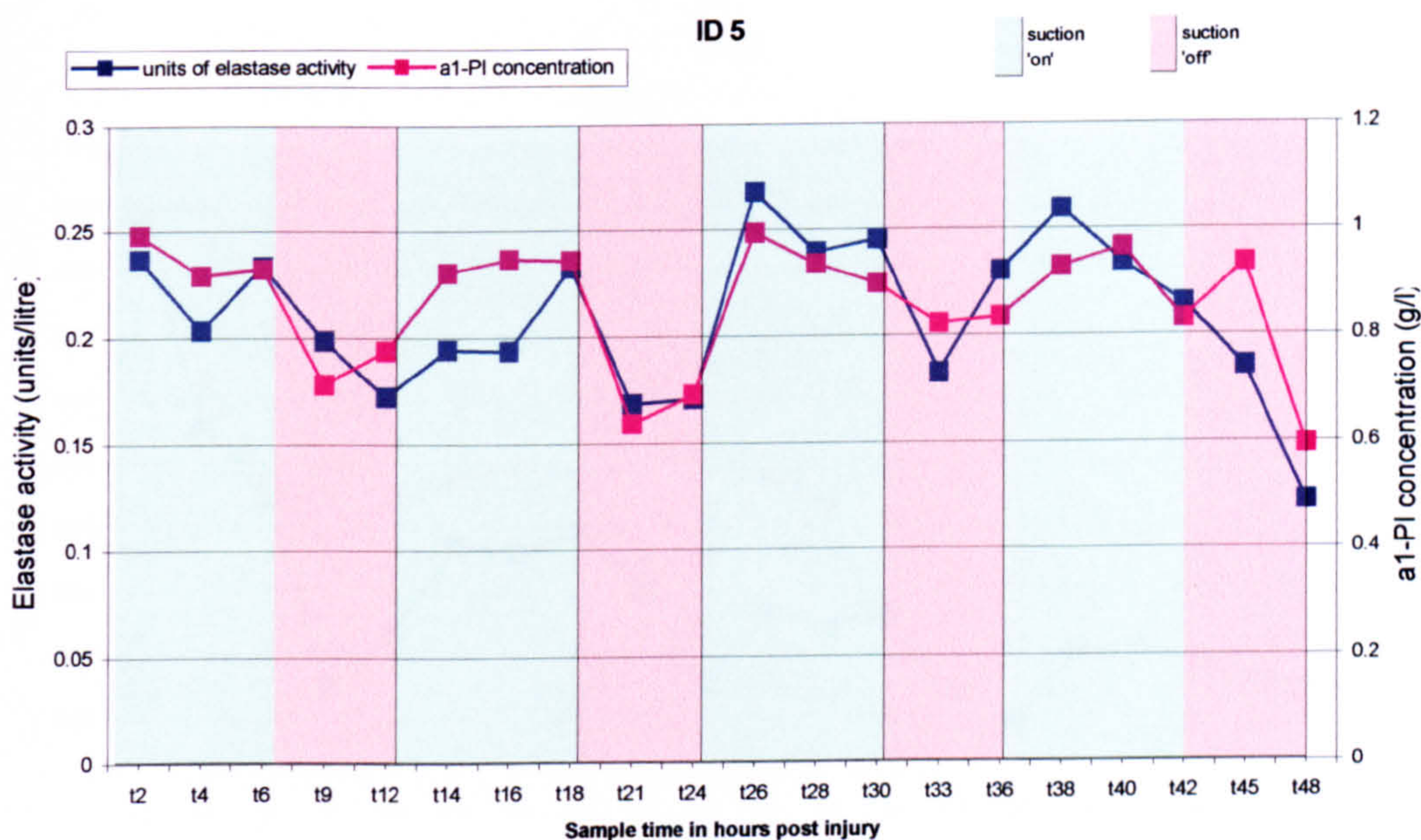
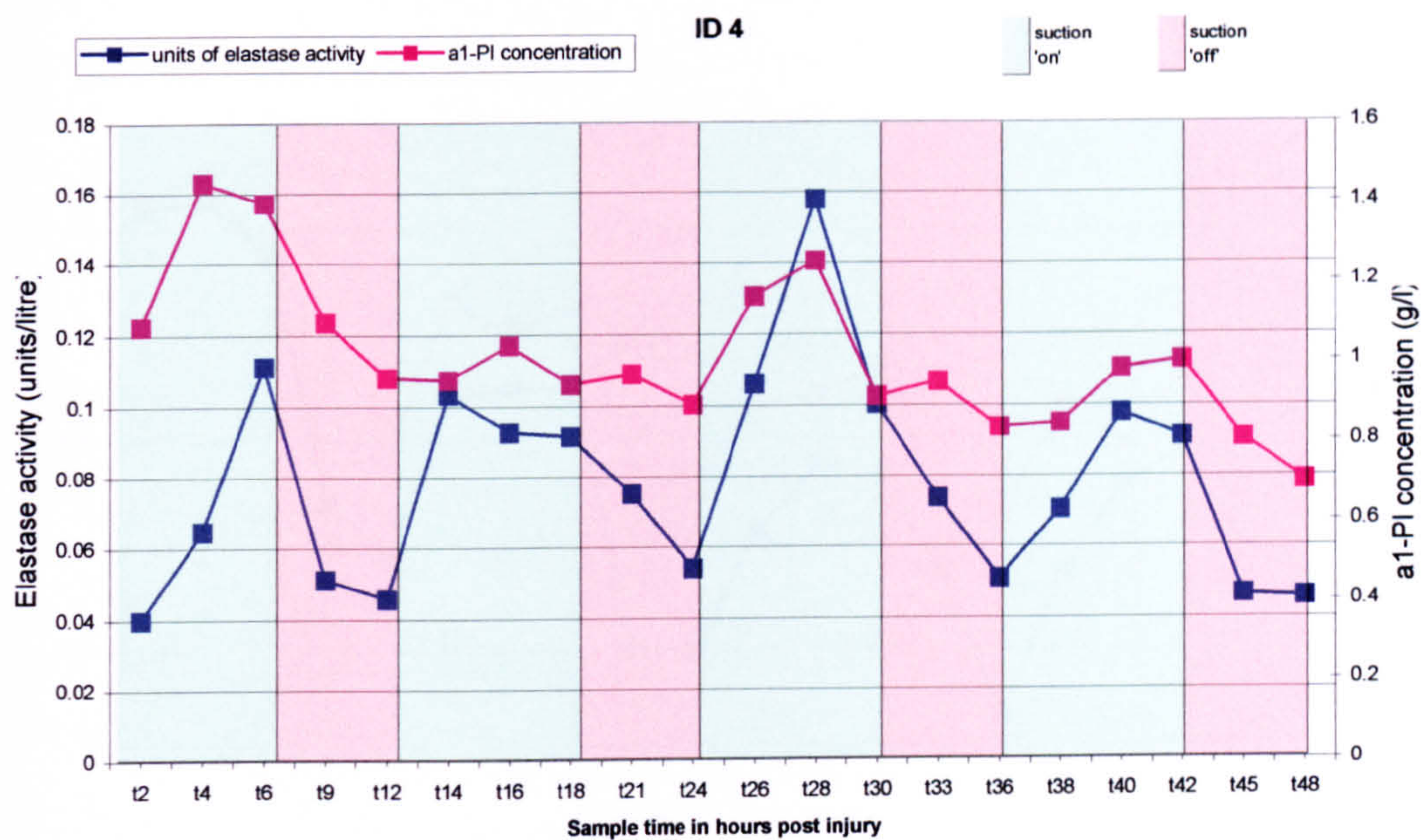
6.3.7 Neutrophil Elastase / α_1 -PI balance in partial thickness burn wound fluid samples

The results of the protease activity determined fluorometrically, and α_1 -PI concentrations are displayed together for each set of burn wound fluids in Figure 55 on page 180. There is also no evidence to suggest that the neutrophil elastase/ α_1 -PI balance is altered during the on/off phases of suction. Again, the relative levels of the two enzymes mirror each other closely, with the ratios of the protease and anti-protease showing no differences ($P=0.202$).

When the same sets of graphs are displayed for the protease activity determined colourimetrically, with α_1 -PI concentrations (as shown in Figure 56 on page 183) the results are less clear, although a similar pattern emerges during on/off suction.

Figure 54 Combined elastase activity (fluorometric) and α_1 -PI concentration in donor site wound fluid samples





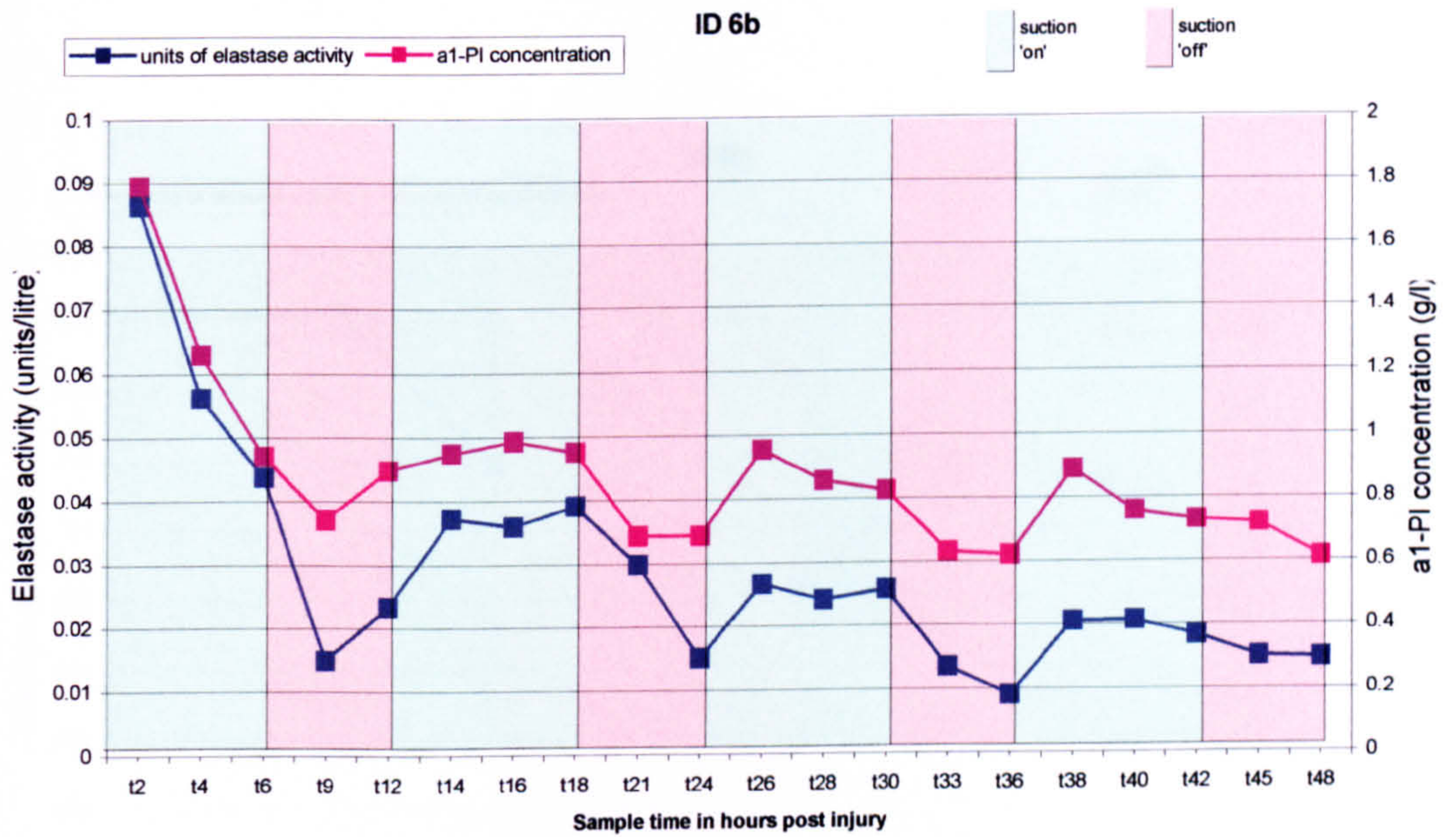
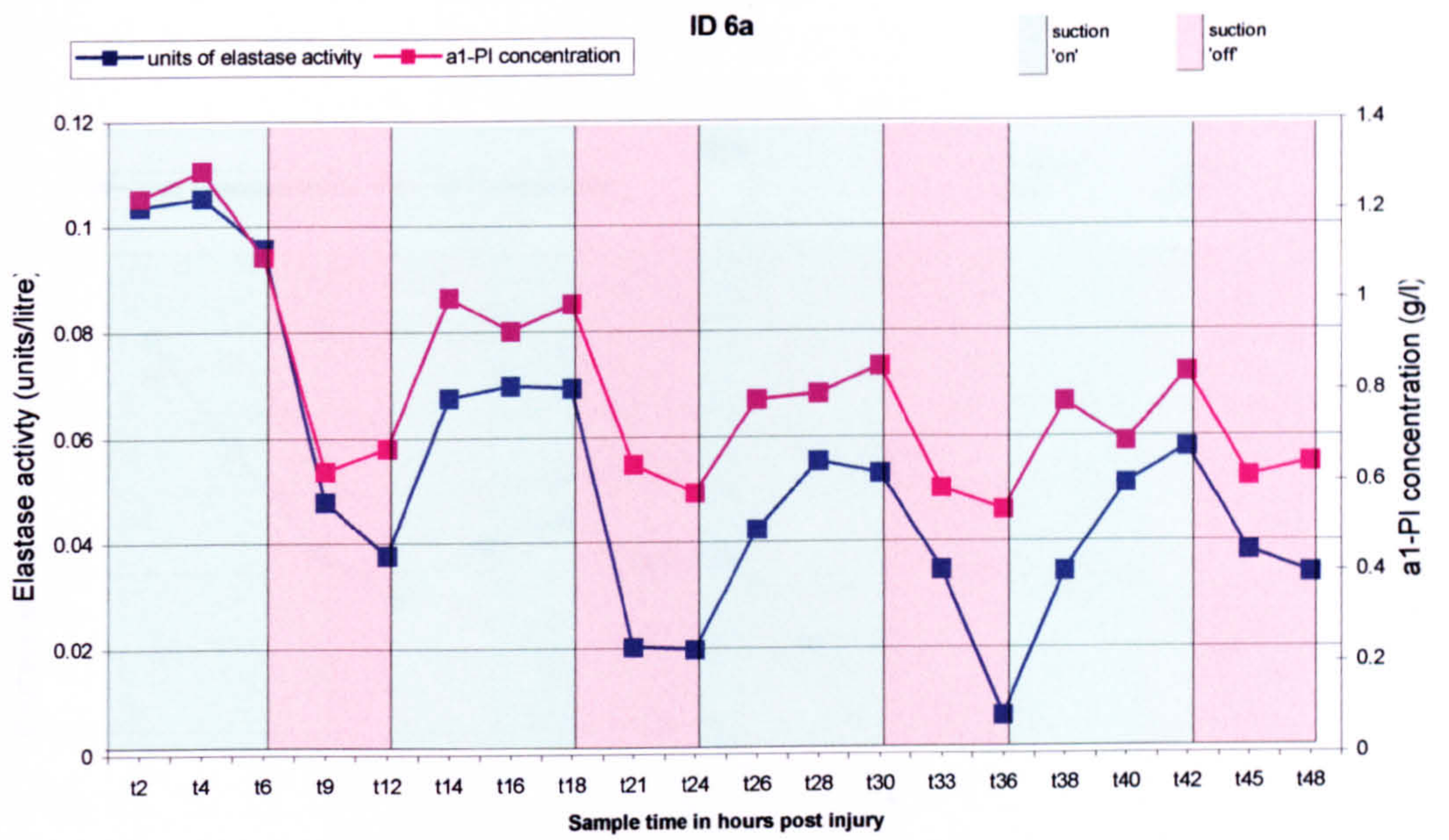
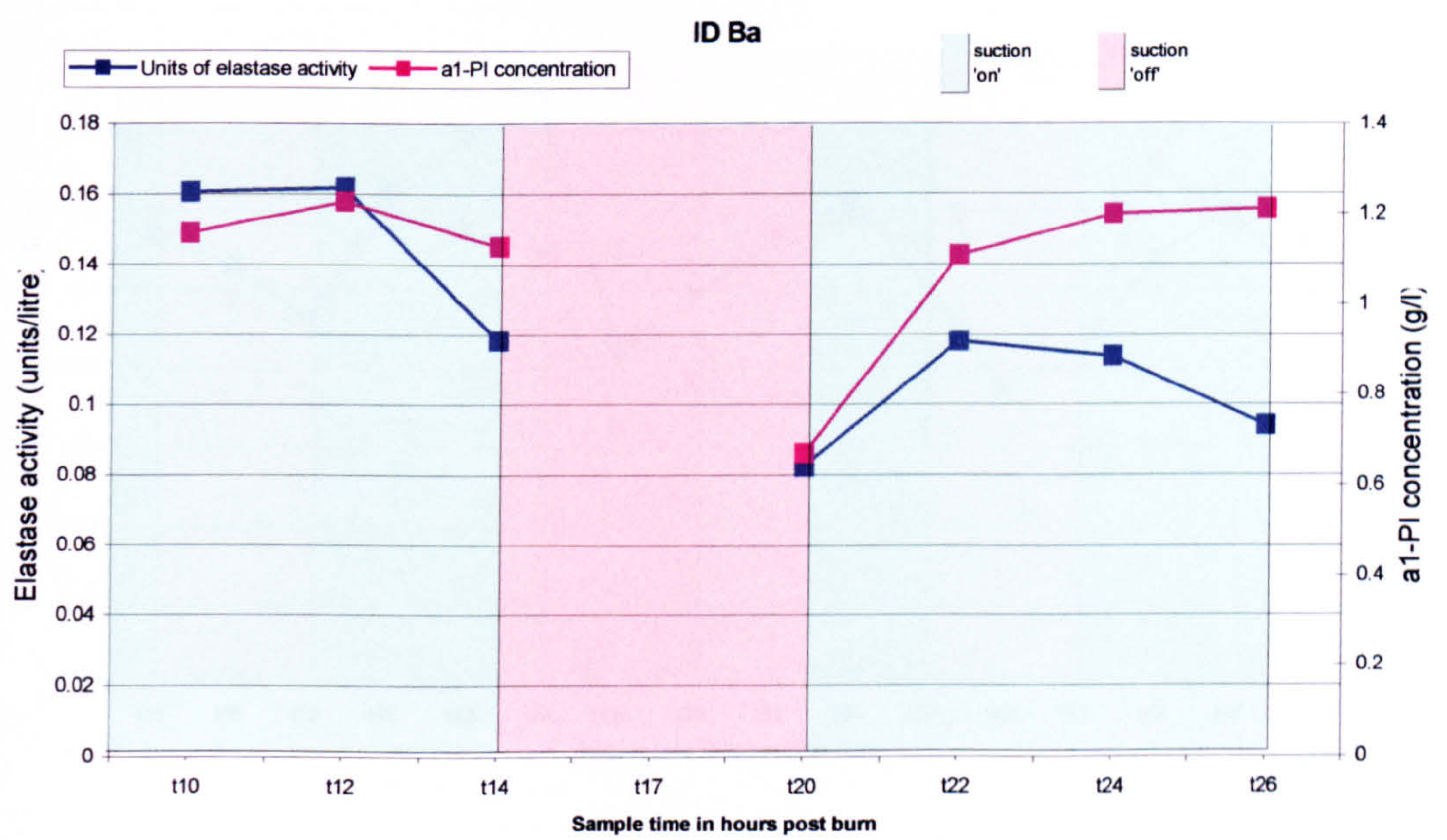
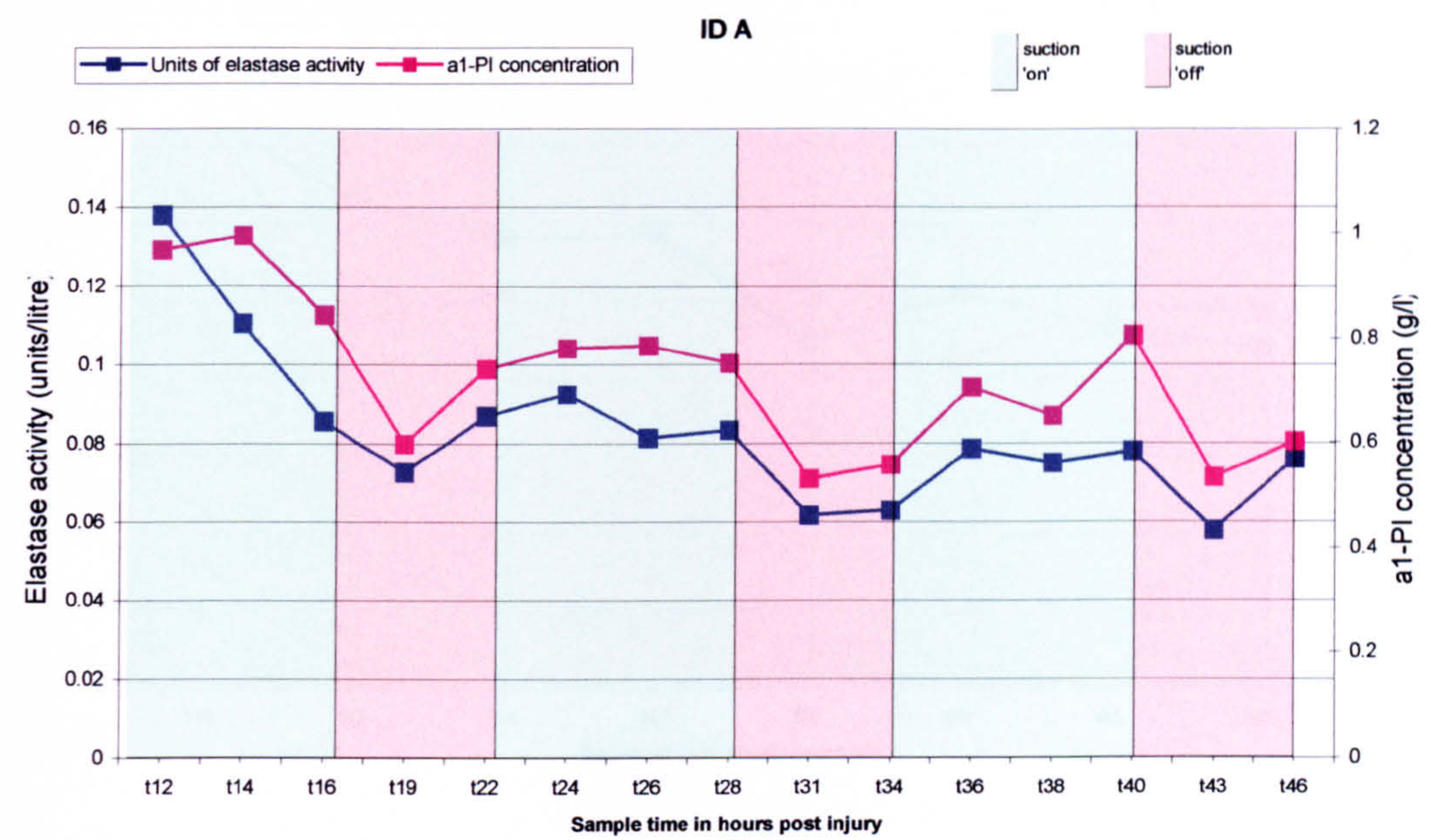
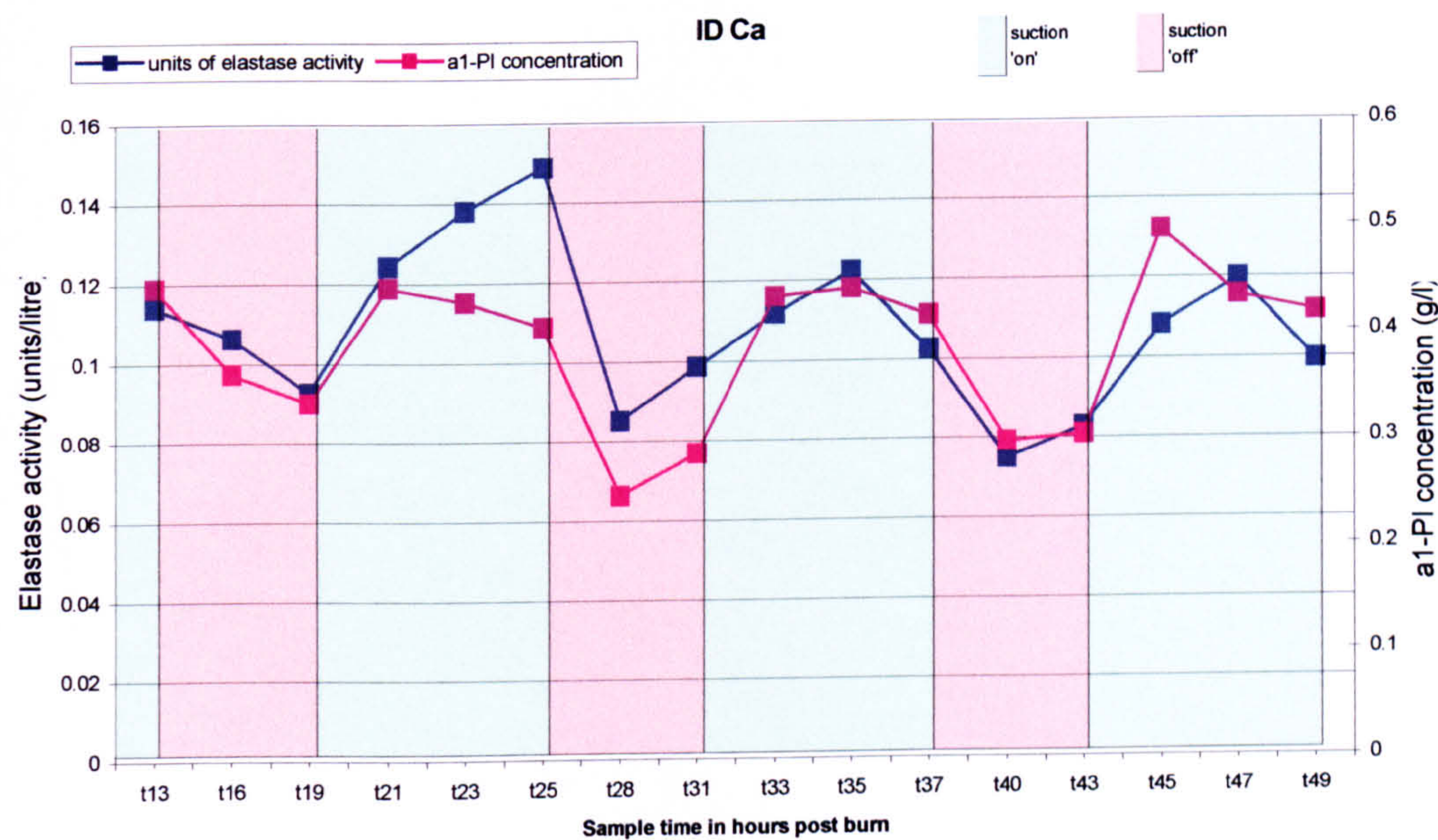
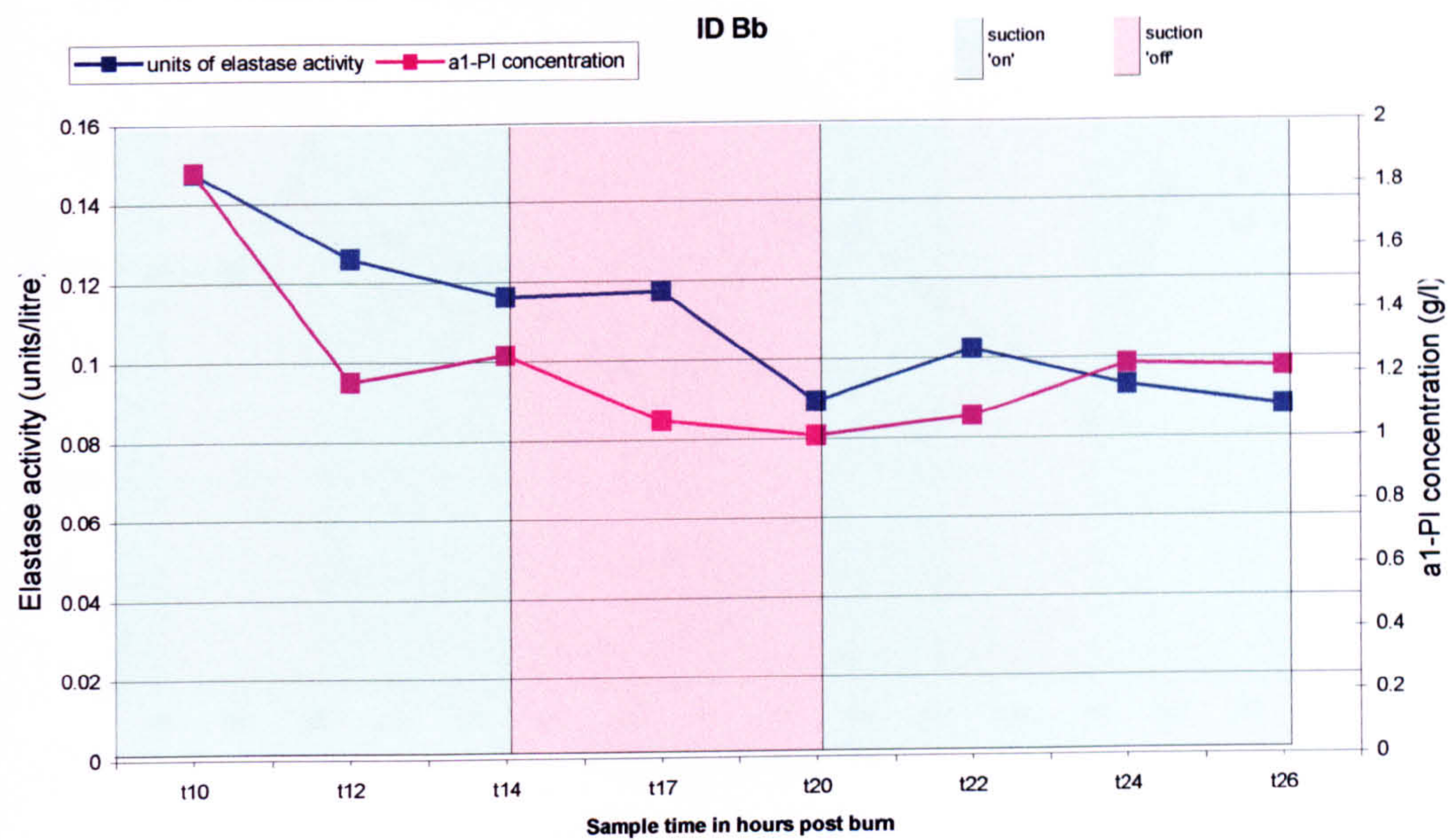


Figure 55 Combined elastase activity (fluorometric) and α_1 -PI concentration in partial thickness burn wound fluid samples





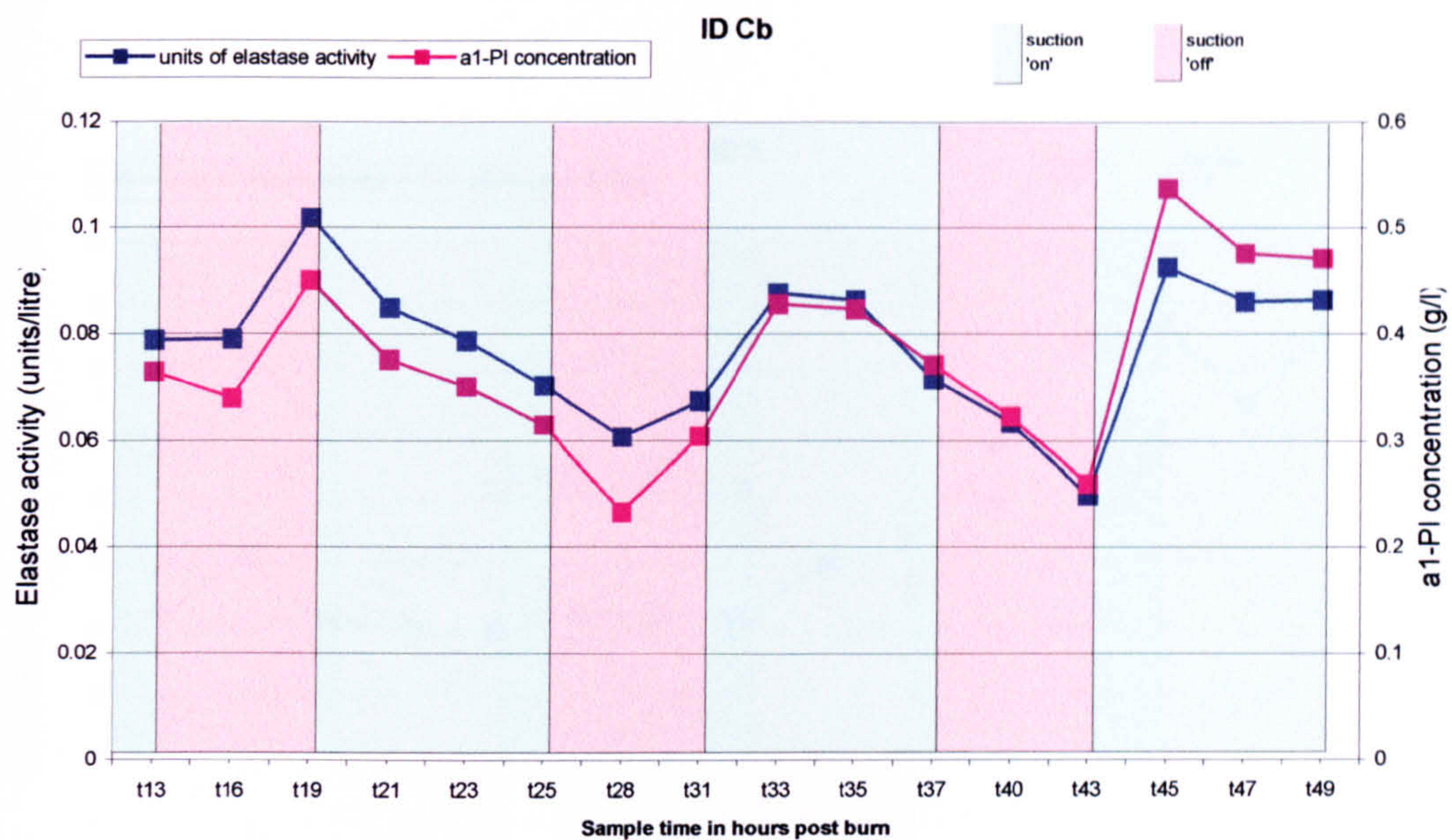
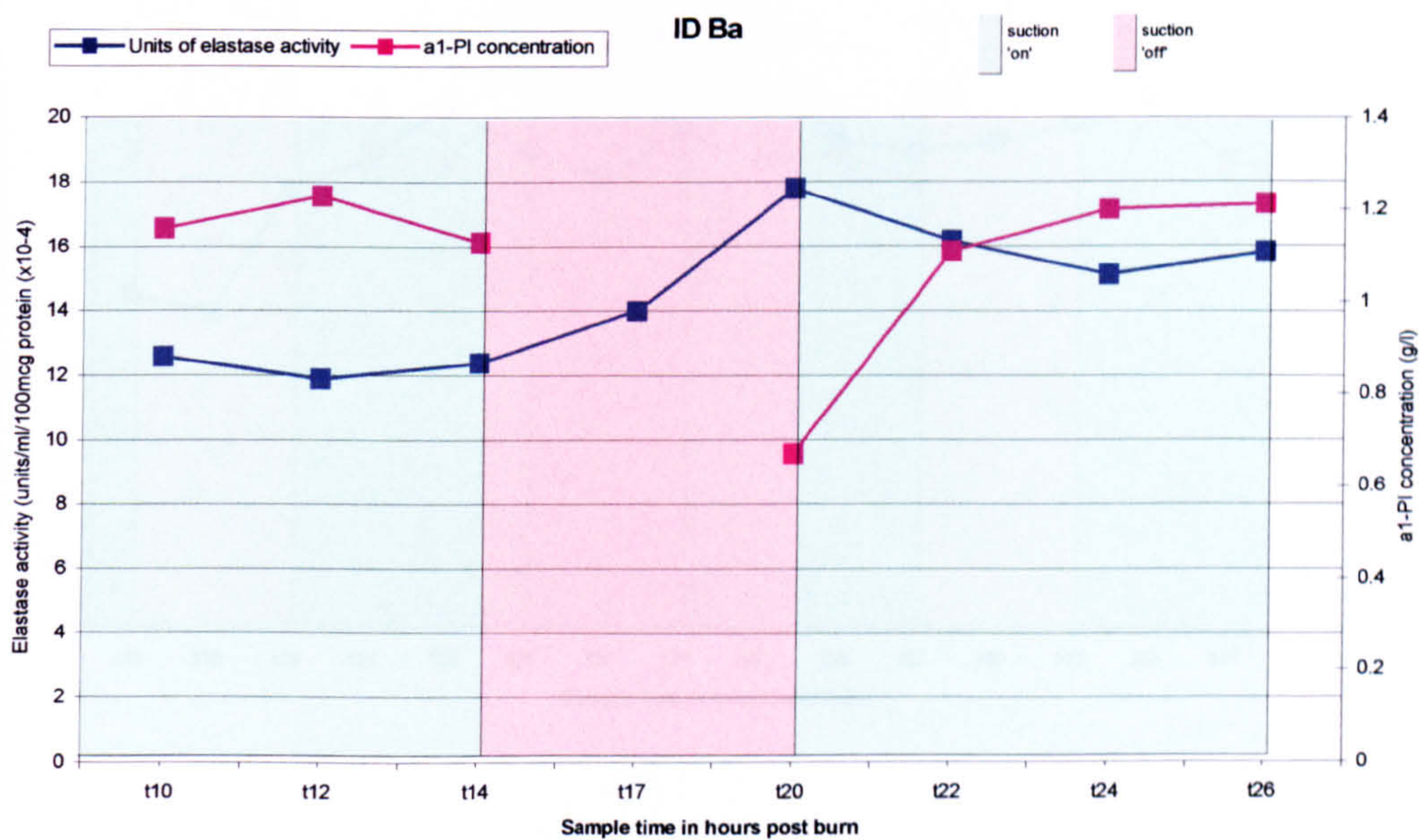
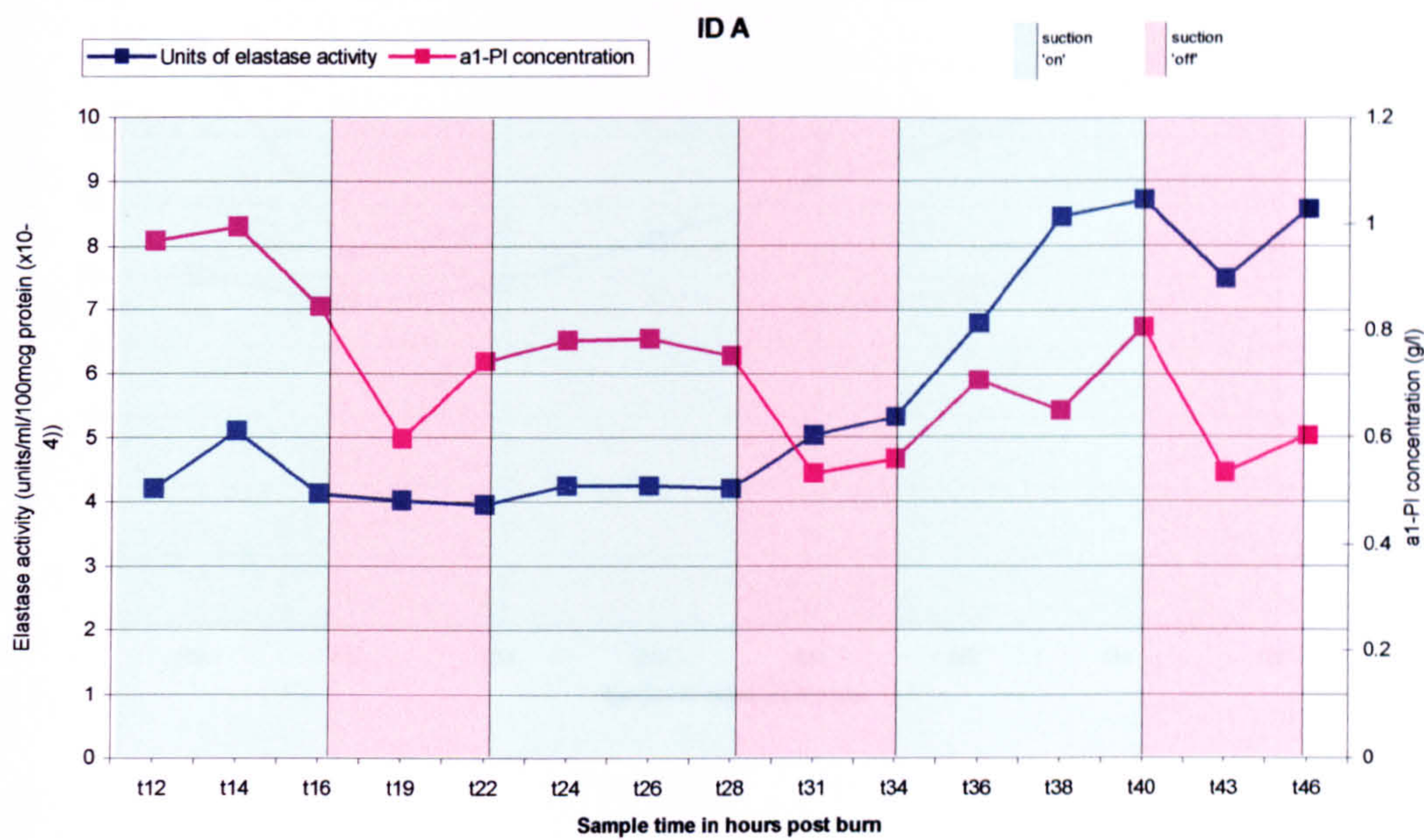
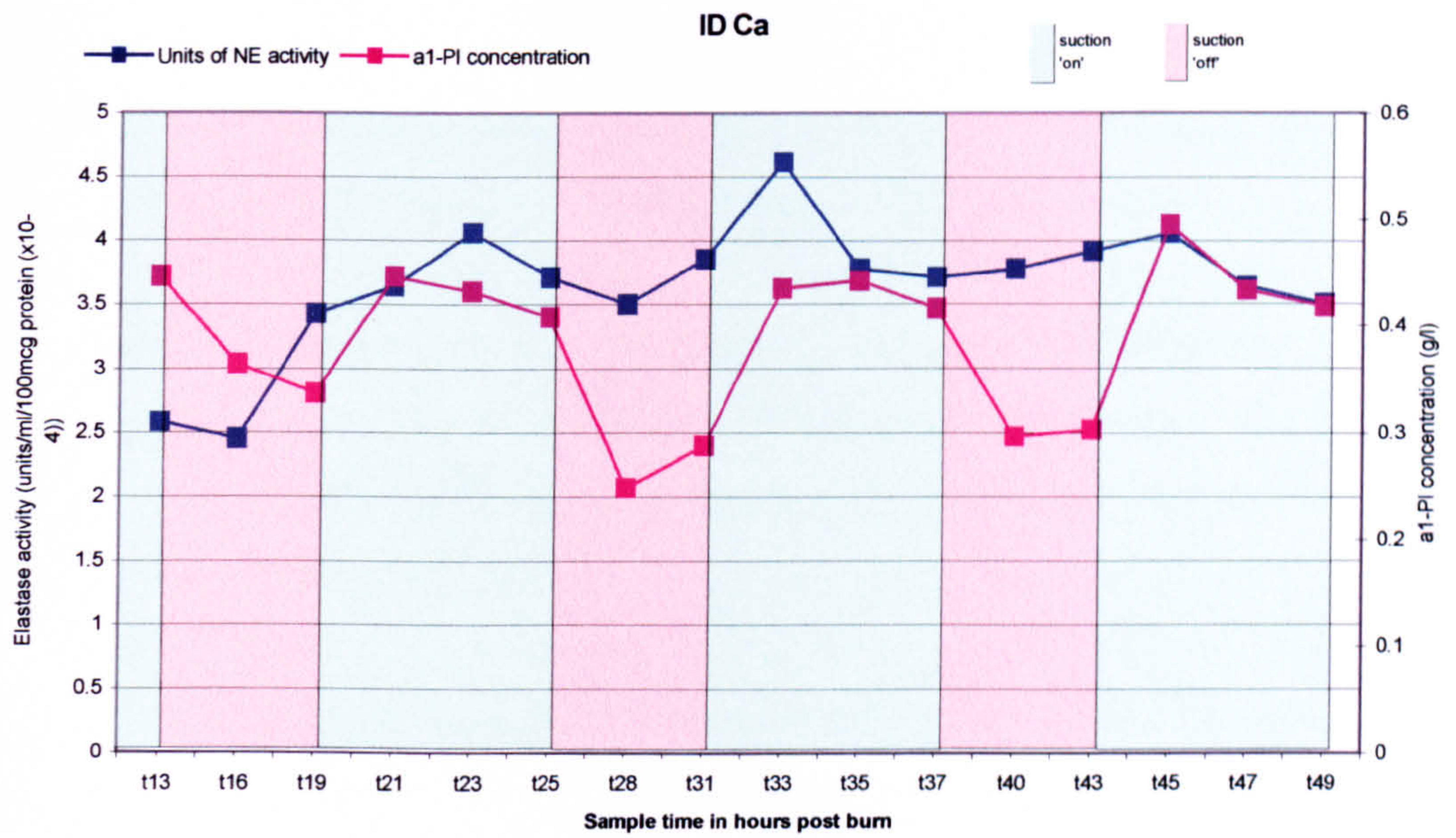
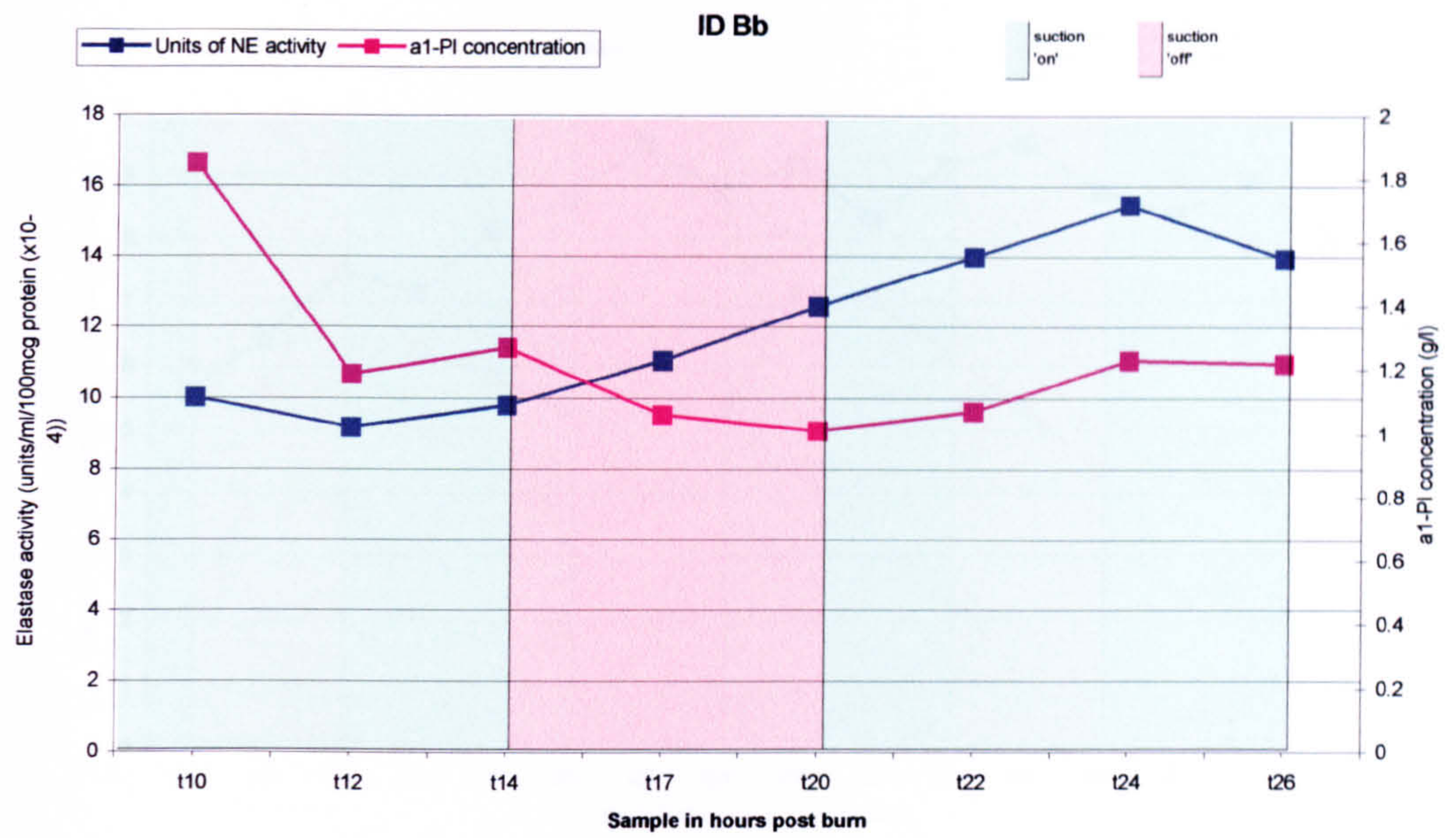
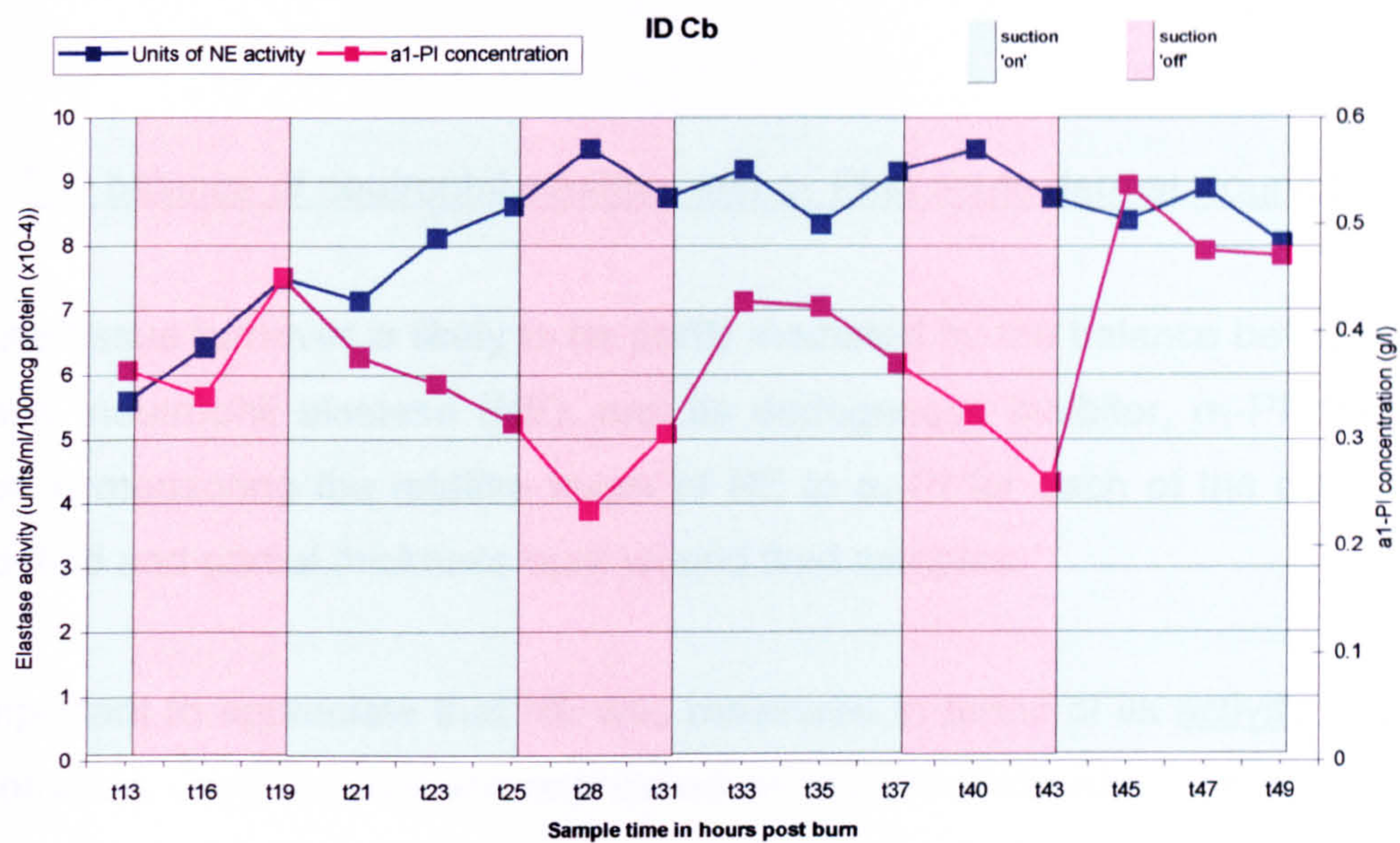


Figure 56 Combined elastase activity (colourimetric) and α_1 -PI concentration in partial thickness burn wound fluid samples







6.4 Discussion

6.4.1 The balance of neutrophil elastase and α_1 -PI in acute dermal wound fluids

Because tissue turnover is likely to be partly mediated by the balance between the protease, neutrophil elastase (NE), and its endogenous inhibitor, α_1 -PI, there is interest in measuring the relative levels of NE to α_1 -PI for each of the donor site wound fluid and partial thickness burn wound fluid samples.

It is important to appreciate that NE was measured in terms of its activity and not concentration. α_1 -PI values are expressed as relative concentrations and not as activity. There was no assay currently available that was able to measure α_1 -PI as units of activity. Hence, displaying the relative ratio of NE activity to α_1 -PI concentration within each fluid sample in each patient, to determine a protease/anti-protease imbalance, is not valid. However, Bullen et al. (1995) have published similar wound fluid data, analysing MMP-9 (Gelatinase B) activity with its endogenous inhibitor concentration, TIMP-1, in surgical drains of five patients who underwent mastectomies. In their study, they compared the relative ratios of MMP-9 activity with the relative concentration of TIMP-1.

The results in this chapter clearly demonstrate that TNP therapy is able to modulate NE activity both in donor site and burn wound fluids. Similar results could be observed for α_1 -PI concentrations, the results of which mirror the total protein data described in Chapter 4.

NE is actively released from degranulating neutrophils locally at sites of tissue injury. α_1 -PI is predominantly a serum-derived acute phase protein that enters the wound during the inflammatory response. It was not possible to determine whether a greater amount of α_1 -PI was being sucked from the wound relative to NE because of the different measurement techniques. However, since TNP is capable of sucking α_1 -PI from the plasma, through the interstitial fluid and onto the wound surface, it is possible that large quantities of α_1 -PI will be significantly augmented during suction. By encouraging α_1 -PI to bath the wound in this way

might alter the 1:1 presence (and therefore potentially the reaction) in favour of α_1 -PI.

However the NE activity assay, which measures 'free' elastase present in the fluid, might not include membrane-bound NE, which has been shown to develop a resistance to inhibition by naturally occurring inhibitors (Owen et al. 1995).

6.4.2 Considerations in assay technique

The NE activities measured by both the colourimetric and fluorometric assay techniques described in this study utilised the substrate, Methoxysuccinyl-Ala-Ala-Pro-Val-pNA, which is specific to neutrophil elastase alone. This substrate is not reactive to the other serine proteases, Cathepsin G or Proteinase 3, also present in azurophilic granules of neutrophils (Nakajima et al. 1979). However, these assays cannot discriminate between NE that might have been released by a small sub-population of macrophages (Owen et al., 1994), which might also contribute to the measured elastase activity in the wound fluid samples. However, an assumption has to be made that any possible contribution by macrophages to NE activity measured in these assays is minimal; the majority released from activated neutrophils. Moreover, this study is interested in the relative activities of NE within the same wound, and one might assume the same levels of macrophage activity is present during the first 48 hours.

The wound fluid samples from each patient were assayed in the same batch and variables such as pH, temperature, timing of recordings, and concentration of solutions remained the same for each of the wound fluid samples within each set. In this way, all of these variables were kept constant.

Despite reading the activity at a wavelength of 405nm, the colourimetric assay was unable to dissociate between the pink/red colours observed in the diluted donor site samples. This colour is likely to be due to haem pigments liberated from red blood cells obtained during the collection process by the TNP device. It was noted that the activity readings measured in the donor site assay were giving erroneously large results because of the coloured nature of the samples. Haemoglobin and its pigments have a wide range of absorption spectra varying

from 400 to 640nm. Confirmation that the coloured pigments within the samples might be contributing towards the high absorption measurements obtained was made by omitting the colourimetric substrate to a series of donor site wound fluid samples. The results showed very high readings on the spectrophotometer, which corresponded to a number of different pink/red coloured samples in the wells. This problem was not evident in the burn wound fluid samples since they were not contaminated by blood. However, it is also possible that both types of wound fluid affected the colourimetric assay in other ways. For example, even the burn wound fluid samples were never very clear during absorption readings, often having a faint yellow colour to them despite centrifugation. This alone might explain the less consistent results in this assay compared to the more sensitive fluorometric assay technique.

6.4.3 Neutrophil Elastase activity in donor site and burn wound fluids

The literature review in this thesis was unable to find any study that compared protease activity in donor site wound fluid and burn wound fluid directly. The nearest comparative study to the one in this chapter is that of Neely et al. (1997), who examined the proteolytic activity in 20 homogenised paediatric burn wound tissue samples and harvested donor site tissue. They determined that MMP activity was greater in burn tissues than donor site or control skin. This thesis did not examine proteolytic activities within biopsy specimens. Other studies have shown greater NE activity in wound fluids from chronic cutaneous wounds than acute (non-cutaneous) wounds (Grinnell and Zhu, 1996; Hoffman et al., 1998). Rao et al. (1995), using the same colourimetric assay described above, also showed that NE activity was greater in chronic wound fluid than donor site wound fluid (Rao et al., 1995).

The results described in this chapter do not support the view that NE activity is greater in burn wound fluid than acute donor site wound fluid, suggesting that these two wounds are much the same in terms of their proteolytic activities. Nonetheless, this inconsistency might be confirmed by testing the fluid samples for MMP activity, for a greater amount of work has been published on these proteases. Despite the similar pathophysiological proteolytic mechanisms that

might be occurring in burn wound and chronic wound fluids, the similar levels of NE activity in acute burn wound and acute donor site wound fluid measured in this study does not support the concept that burn wounds behave like chronic wounds. Nonetheless, although this study measured protease activity in wound fluid, this may not necessarily reflect protease activity in the tissues directly.

Despite the compelling evidence that connective tissue proteolysis is occurring in inflamed tissues, in the presence of increased NE activity, does not prove that neutrophil-derived NE is responsible; the injury might be produced, in part, by other proteases released from other cells, and that the measurable increases in NE activity, for example, are simply a reflection of this.

It must be emphasised that this thesis neither provides evidence to demonstrate that neutrophil elastase directly contributes to burn wound pathophysiology nor attempts to establish whether TNP therapy might improve burn wound healing. Instead, this study does demonstrate that the protease activities can be altered by this novel device, which supports the view that TNP therapy might modulate the protease environment of the wound to favour healing.

6.5 Conclusion

This chapter support the hypothesis that TNP therapy is capable of modulating the protease activity of acute dermal wounds by altering the activity within the wound fluid environment. These conclusive findings might explain one mechanism of action by which TNP might improve the healing of some difficult-to-heal wounds, by actively removing proteases from the wound environment. The effects of TNP on the inhibitor of Neutrophil Elastase, α_1 -PI, is also shown. This suggests that TNP is capable of drawing the NE inhibitor from the intravascular system through the wound and onto the surface. However, this study fails to establish whether a true protease/anti-protease imbalance might be occurring during intermittent TNP therapy since protease activity and anti-protease concentrations cannot be readily compared.

7 SUMMARY

The first aim of this thesis was to develop a method of applying selective TNP to dermal wound surfaces (based on commercially available V.A.C.[™] dressings) to allow, for the first time, an opportunity to study its effects using intra-patient controls. Chapter 3 explained how this was achieved and provided evidence to show that the TNP device was capable of mimicking normal V.A.C.[™] dressings. A further device was developed as a research tool that enabled the device to collect wound fluid samples.

To test the hypothesis that TNP (V.A.C.[™]) therapy might be capable of modulating the wound fluid constituents of a wound, chapter 4 described how the irrigation TNP device was used to collect wound fluid intermittently on the same wound, using the 'off' phase of collection as a control. On both donor site and burn wounds, the levels of total protein mirrored the intermittent phases of suction, thereby providing evidence that TNP might indeed alter the wound environment by its behaviour on wound fluid.

Chapter 5 provided evidence that, not only wound fluid, but also the presence of inflammatory cells might also be influenced by TNP therapy. Neutrophil cell counts performed on paired TNP-treated and control wound biopsy sections were compared in both donor site and burn wounds. The results showed that TNP therapy was capable of significantly reducing neutrophil cell numbers compared to controls in donor site wounds with similar trends in burn wounds, although this was not significant. Furthermore, TNP therapy was capable of reducing the distribution of cells for up to 1mm beneath the surface of the wound.

The pathophysiological role of neutrophil-derived proteases in mediating tissue damage in difficult-to-heal wounds and ways in which TNP therapy might be able to alter this was tested in chapter 6. On the same set of wound fluid samples obtained in chapter 4, neutrophil elastase activity was determined on both donor site and burn wound fluids using two different activity assay techniques. The endogenous inhibitor to neutrophil elastase, α_1 -PI, was determined by nephelometry.

Further evidence that intermittent TNP therapy is capable of modulating the wound fluid environment was also demonstrated by showing that the activity of neutrophil elastase was directly related to the on/off phases of suction. This might explain one of the possible mechanisms by which TNP therapy promotes healing of some chronic wounds in vivo. Given the constraints of measuring protease activity and α_1 -PI concentrations in wound fluids, direct comparisons between the balance of the protease and its inhibitor could not be made. This chapter also demonstrated that there was no significant difference between the neutrophil elastase activity of donor site and burn wound fluids over a 48-hour period.

8 CONCLUSION

- 1. A TNP device was developed to provide selective subatmospheric pressure to cutaneous wounds**
- 2. Validation studies confirmed that the TNP device mimics V.A.C™ dressings in a clinical setting**
- 3. An irrigation TNP device was developed as a research tool to allow frequent and instantaneous collections of wound fluid in vivo**
- 4. TNP therapy was capable of altering the constituents of wound fluid, including proteases, in acute dermal wounds**
- 5. TNP therapy was able to reduce the number of neutrophils present in donor site and partial thickness burn wounds**

9 FUTURE WORK

The TNP device described in this thesis is capable of generating future research work in the following areas:

1. Comparing the effects of TNP therapy on acute or chronic wound healing in intra-patient clinically controlled trials to establish whether a true difference between treated and control groups can be observed or measured
2. To further investigate other putative mechanisms of action that have been ascribed to TNP (including measurements of dermal oedema, bacterial content, granulation tissue formation and mechanical influences of TNP on cells)
3. As a research tool, the irrigation TNP device can be used in the collection of wound fluid during wound healing studies (especially those of chronic wounds)

1. Intra-patient clinically controlled trials

A number of intra-patient clinical trials on different wound types, including acute, chronic and burn wounds are now possible using the TNP device to test whether TNP-treated wounds might heal more quickly than controls. Only two randomised controlled studies have been published describing its efficacy in chronic wounds, and these have been conducted in between-patient groups. Applying two TNP devices to the same wound type, one under suction and the other simply as a control, might be used to establish a true clinical benefit. For example, the rates of reepithelialisation in donor site wounds (and other wounds) can be determined using this selective TNP device. Wound biopsies can be harvested from TNP-treated areas and compared to control areas after 7-10 days.

The use of the TNP device with portable suction pumps does not necessarily limit these trials to the bedside.

2. Testing other suggested mechanisms of action of TNP

One of the proposed beneficial effects of TNP may be due to the attenuation of oedema present in some wounds. Many investigators have suggested that oedema may be responsible for the delay in wound healing by limiting the blood flow to the wound. Using the donor site wound model, some preliminary work has been performed using high frequency (20MHz) ultrasound to measure directly the thickness of the dermis in TNP-treated versus control sites. There is some evidence that TNP therapy reduces the thickness of the dermis but this work requires more study using this device.

The effects of constant versus intermittent suction can also be evaluated in a number of ways. Laser Doppler imaging of the skin comparing both modes of action on dermal blood flow can be determined. There is some evidence that intermittent (5 minutes on / 2 minutes off) therapy may be more beneficial to wound healing than constant suction alone. Measuring the protease profiles from samples taken using each of these modes of therapy may also determine whether constant or intermittent therapy may be better at removing proteases during wound healing.

The effects of mechanical loading by TNP therapy on wounds could also be addressed using selective TNP therapy. There is evidence that tissues respond differently to changes in mechanical loads (for example in granulation tissue formation and keratinocyte proliferation).

3. The TNP device as a research tool in wound healing studies

The irrigation TNP device could be used specifically as a research tool to obtain wound fluid samples from any cutaneous wound surface in the temporal analysis of a number of wound fluid parameters. For example, other neutral proteases (including the metalloproteases, MMP-8 and MMP-9) may be measured for protease activity and compared directly to serine proteases (such as neutrophil

elastase) to determine the relative temporal levels of expression in a number of wounds and during different stages of healing. The device might also be used to establish the relative levels of other wound constituents, including local cytokine and growth factor production in different wound types. In these cases, the device could be placed on a wound surface as a simple dressing (without suction) and switched on at various times just to retrieve wound fluid from the wound and not as a treatment per se. The advantage of this system as a research tool is in its ability to obtain wound fluid as frequently as desired, easily and reliably. Importantly, the collection method can be achieved over minutes and not hours.

The irrigation TNP device might also be used to apply topical nutrition or growth factors (such as TGF- β) to a wound surface as part of a drug release system for topical wound treatment.

10 APPENDIX

10.1 Data sheet for recording times and fluid volumes from donor site wounds

10.2 Data sheet for recording times and fluid volumes from burn wounds

10.3 Materials used in the biochemical assays

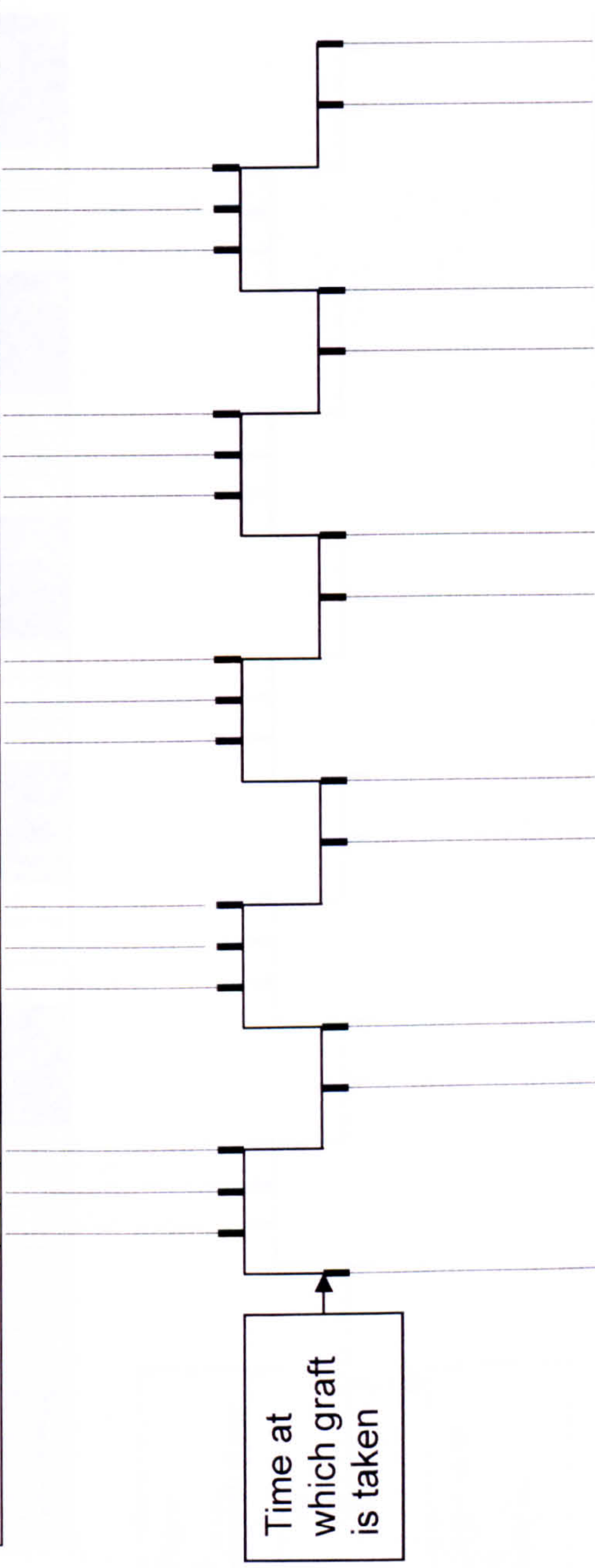
10.4 Protocols for histological staining

10.5 Protocol for immunohistochemical staining for neutrophil elastase

10.6 Cell counting data for donor site wound biopsies

10.7 Cell counting data for burn wound biopsies

10.1 Data sheet for recording times and fluid volumes from donor site wounds

[illegible][illegible]

10.2 Data sheet for recording times and fluid volumes from burn wounds

[illegible]

10.3 Materials used in the biochemical Assays

(see text for details)

A. 0.1M HEPES buffer (pH7.4)

100ml of 0.1M HEPES buffer was prepared by mixing 2.38gm HEPES (Sigma Chemical UK.) and 2.92gm NaCl (0.5M) with 100mls ddH₂O. The pH was calibrated to 7.4.

B. BCA working reagent

Reagent A (hydroxide) includes sodium carbonate, sodium bicarbonate, BCA detection reagent and sodium tartrate in 0.1M NaOH

Reagent B (Cu²⁺) includes 4% CuSO₄ in distilled water.

C. Tris-HCl buffer

0.2M Tris-HCl was prepared by mixing 2.422g Trizma Base and 5.844g NaCl up to 100ml ddH₂O. The pH was calibrated to 8.5.

D. 7-AMC standard

For a 1mM solution, 0.0876g of standard is added to 500ml of acetone.

10.4 Protocol for immunohistochemical staining for Neutrophil Elastase

The following protocol was used for both donor site and partial thickness burn biopsy sections

1. Sections placed in an oven at 60°C for 30 minutes
2. De-wax in 2 changes of xylene for 3 minutes each
3. Rehydrate in graded alcohols (100%, 100%, 70% for 1 minute each)
4. Block endogenous peroxidase with 3% hydrogen peroxide in methanol for 20 minutes
5. Wash in running tap water for 5 minutes
6. Perform antigen retrieval using 0.1% trypsin digestion method at 37°C for 30 minutes in a humidity chamber (see Trypsin preparation on page 202)
7. Rinse sections with PBST
8. Ring around sections with a hydrophobic pen (Dako, UK)
9. Incubate sections with 10% Normal Swine Serum (NSS, Dako, UK) in PBS for 10 minutes to reduce non-specific background staining for polyclonal primary antibodies
10. Rinse off NSS with PBST
11. Apply NP57 (Dako, UK) primary antibody (optimally diluted in PBS) for 30 minutes covered from direct sunlight in a humidity chamber
12. Rinse with three 3 minute changes of PBST
13. Apply the Dako EnVision™+ reagent for 30 minutes (see page 139)
14. Rinse with three 3 minute changes of PBST
15. Cover the sections with freshly prepared diaminobenzidine solution (DAB, Dako, UK)
16. Stop the reaction with PBS after 10 minutes
17. Wash in running tap water for 5 minutes
18. Counterstain with Mayer's haematoxylin for 3 minutes
19. Blue in water for 5 minutes in water bath
20. Dehydrate in graded alcohols (70% for 15 seconds, 2 changes of 100% for 30 seconds)
21. Clear in two 1 minute changes of xylene
22. Mount slides in DPX and leave to dry for 24 hours

Antigen retrieval with Trypsin digestion:

Formaldehyde fixation leads to cross-linking “methylene bridges” between proteins in the tissues. Subsequent processing to paraffin wax in these biopsies can further mask the antigenic epitopes, thereby reducing the sensitivity of the immunocytochemical labelling technique. These masked antigens can be retrieved using a proteolytic (trypsin) digestion technique, in which the formaldehyde crossing-linking is broken down and the antigenic sites uncovered. Under-digestion results in very little staining of the primary antibody. Similarly, over-digestion can lead to tissue damage.

Using the manufacturers’ instructions, preparation of the trypsin solution proceeded as follows:

- A. 0.1% CaCl₂ (pH 7.8) was prepared by dissolving 50mg CaCl₂ (BDH Laboratory Supplies, UK) in 50mls of 0.05mol/l Tris/HCl, 0.15mol/l NaCl, pH 7.8.
- B. 2% trypsin stock solution was prepared by dissolving the contents of one 500mg vial of DAKO trypsin (Code No. S2012) in freshly prepared 0.1% CaCl₂, pH 7.8
- C. A 0.1% trypsin working solution was prepared from aliquots of stored 2% stock solution mixed with 0.1% CaCl₂, pH 7.8 as described in the manufacturers’ instructions. This working solution was used in the protocol described above at an optimal time of 30 minutes based upon previous experience at the Stoke Mandeville Burns & Reconstructive Surgery Research Trust.

10% Normal Swine Serum (NSS)

10% NSS (Dako, UK) was prepared by mixing the stock solution with PBS. This was then brought up to 37°C prior to use in the above protocol.

Monoclonal mouse anti-human Neutrophil Elastase (NP57)

The primary monoclonal NP57 antibody (Dako, UK. Code No. M0752) was optimally diluted (1:120) with PBS.

Phosphate buffered saline (PBS) with TWEEN (PBST)

Standard PBS was prepared by dissolving 1 tablet (Oxoid, Ltd, UK) in 100mls of ddH₂O. Up to 10 litres of PBS was prepared, much of it being used with TWEEN as a wash buffer (see below). PBS was stored at 2°C and used with 3 days.

For the wash buffer, 0.05% TWEEN detergent (courtesy Mr. K. Miller, Department of Histopathology, University College London) was added to PBS. The use of TWEEN helps avoid excessive background staining.

Dako EnVision™+ System, Peroxidase (DAB)

This commercial product, purchased as a kit from Dako (K4006), containing both the peroxidase labelled polymer and the DAB substrate-chromogen solution was used in accordance to the manufacturers' instructions.

10.5 Cell counting data for donor site wound biopsies

Donor ID 1

1 toCON	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	2	1	1	1	0	0	0	5	0.71
hpf2	0	0	0	0	0	0	0	0	0.00
hpf3	0	0	0	0	0	0	0	0	0.00
hpf4	0	0	0	0	0	0	0	0	0.00
hpf5	0	0	0	0	0	0	0	0	0.00
hpf6	0	0	0	0	0	0	0	0	0.00
hpf7	0	0	0	0	0	0	0	0	0.00
hpf8	0	0	0	0	0	0	0	0	0.00
hpf9	0	0	0	0	0	0	0	0	0.00
hpf10	0	0	0	0	0	0	0	0	0.00
total	2	1	1	1	0	0	0	5	0.71
average	0.2	0.1	0.1	0.1	0	0	0	0.5	0.07

1 t48CON	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	27	38	52	54	61	32	58	322	46.00
hpf2	3	24	28	14	5	62	20	156	22.29
hpf3	3	12	16	36	9	15	5	96	13.71
hpf4	3	49	23	3	2	2	0	82	11.71
hpf5	4	21	28	3	3	0	3	62	8.86
hpf6	2	6	30	7	3	1	1	50	7.14
hpf7	0	4	4	10	0	0	0	18	2.57
hpf8	0	2	8	5	0	2	13	30	4.29
hpf9	1	2	0	0	0	13	8	24	3.43
hpf10	0	0	0	2	6	3	3	14	2.00
total	43	158	189	134	89	130	111	854	122.00
average	4.3	15.8	18.9	13.4	8.9	13	11.1	85.4	12.20

1 t48TNP	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	25	20	33	47	25	36	45	231	33.00
hpf2	0	19	12	26	15	35	61	168	24.00
hpf3	0	5	6	29	23	19	54	136	19.43
hpf4	0	3	2	8	20	10	10	53	7.57
hpf5	2	4	0	1	5	10	2	24	3.43
hpf6	1	0	4	1	2	0	3	11	1.57
hpf7	0	2	1	1	1	1	0	6	0.86
hpf8	0	0	0	1	6	0	0	7	1.00
hpf9	0	0	0	0	0	0	0	0	0.00
hpf10	0	0	0	3	0	0	0	3	0.43
total	28	53	58	117	97	111	175	639	91.29
average	2.8	5.3	5.8	11.7	9.7	11.1	17.5	63.9	9.13

Donor ID 2

2 toCON	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	1	0	1	1	3	0	0	6	0.86
hpf2	0	0	0	0	0	0	1	1	0.14
hpf3	1	0	0	0	1	0	0	2	0.29
hpf4	0	0	0	0	0	0	0	0	0.00
hpf5	0	1	0	0	0	0	0	1	0.14
hpf6	1	0	0	0	0	0	1	2	0.29
hpf7	2	0	0	0	0	0	0	2	0.29
hpf8	1	0	0	0	0	0	0	1	0.14
hpf9	0	0	0	0	0	0	0	0	0.00
hpf10	0	0	0	0	0	0	0	0	0.00
total	6	1	1	1	4	0	2	15	2.14
average	0.6	0.1	0.1	0.1	0.4	0	0.2	1.5	0.21

2 t48CON	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	40	56	45	65	45	62	69	382	54.57
hpf2	24	27	9	9	7	6	8	90	12.86
hpf3	18	24	5	3	6	7	0	63	9.00
hpf4	1	7	11	7	5	2	0	33	4.71
hpf5	5	10	14	9	6	1	1	46	6.57
hpf6	1	8	28	15	2	2	2	58	8.29
hpf7	0	6	10	15	5	2	0	38	5.43
hpf8	0	4	2	5	0	3	0	14	2.00
hpf9	0	2	1	3	0	0	0	6	0.86
hpf10	0	0	13	1	0	0	0	14	2.00
total	89	144	138	132	76	85	80	744	106.29
average	8.9	14.4	13.8	13.2	7.6	8.5	8	74.4	10.63

2 t48TNP	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	48	47	41	51	33	47	42	309	44.14
hpf2	2	2	3	8	15	4	3	37	5.29
hpf3	14	4	5	1	5	7	14	50	7.14
hpf4	1	0	2	0	0	1	5	9	1.29
hpf5	1	1	1	1	3	6	0	13	1.86
hpf6	1	0	3	1	2	2	3	12	1.71
hpf7	0	0	1	0	1	2	0	4	0.57
hpf8	0	0	0	0	0	2	3	5	0.71
hpf9	0	0	0	0	0	0	0	0	0.00
hpf10	0	0	0	0	0	0	0	0	0.00
total	67	54	56	62	59	71	70	439	62.71
average	6.7	5.4	5.6	6.2	5.9	7.1	7	43.9	6.27

Donor ID 4

4 toCON	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	0	1	1	1	3	0	0	6	0.86
hpf2	0	0	0	0	1	1	0	2	0.29
hpf3	0	0	0	0	0	0	0	0	0.00
hpf4	0	0	0	0	0	0	0	0	0.00
hpf5	0	0	0	0	0	0	0	0	0.00
hpf6	1	0	0	0	0	0	0	1	0.14
hpf7	1	0	0	0	0	0	0	1	0.14
hpf8	0	0	0	0	0	0	0	0	0.00
hpf9	0	0	0	0	0	0	0	0	0.00
hpf10	0	0	0	0	0	0	0	0	0.00
total	2	1	1	1	4	1	0	10	1.43
average	0.2	0.1	0.1	0.1	0.4	0.1	0	1	0.14

4 t48CON	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	47	90	54	51	50	51	38	381	54.43
hpf2	7	14	5	21	10	6	4	67	9.57
hpf3	1	6	1	2	1	3	3	17	2.43
hpf4	0	3	7	3	1	3	2	19	2.71
hpf5	2	1	5	3	3	1	1	16	2.29
hpf6	2	1	1	3	0	1	0	8	1.14
hpf7	0	2	1	0	0	0	0	3	0.43
hpf8	0	2	0	0	0	0	0	2	0.29
hpf9	0	0	0	0	0	0	0	0	0.00
hpf10	0	0	0	0	0	0	1	1	0.14
total	59	119	74	83	65	65	49	514	73.43
average	5.9	11.9	7.4	8.3	6.5	6.5	4.9	51.4	7.34

4 t48TNP	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	40	30	32	24	23	26	27	202	28.86
hpf2	5	3	8	7	6	4	4	37	5.29
hpf3	0	0	5	2	1	2	0	10	1.43
hpf4	1	2	2	2	1	0	1	9	1.29
hpf5	0	1	0	0	0	0	0	1	0.14
hpf6	0	1	0	0	1	1	1	4	0.57
hpf7	0	0	0	0	0	0	0	0	0.00
hpf8	0	0	0	0	0	0	0	0	0.00
hpf9	0	0	0	0	0	0	0	0	0.00
hpf10	0	0	0	0	0	0	0	0	0.00
total	46	37	47	35	32	33	33	263	37.57
average	4.6	3.7	4.7	3.5	3.2	3.3	3.3	26.3	3.76

Donor ID 5

5 toCON	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	1	3	1	1	1	0	0	7	1.00
hpf2	0	0	1	0	0	0	0	1	0.14
hpf3	0	0	0	0	0	0	0	0	0.00
hpf4	0	0	0	0	0	0	2	2	0.29
hpf5	1	0	0	0	0	0	0	1	0.14
hpf6	0	0	0	0	0	0	0	0	0.00
hpf7	0	0	0	0	0	0	0	0	0.00
hpf8	0	0	0	0	0	0	0	0	0.00
hpf9	0	0	0	0	0	0	0	0	0.00
hpf10	0	0	0	0	0	0	0	0	0.00
total	2	3	2	1	1	0	2	11	1.57
average	0.2	0.3	0.2	0.1	0.1	0	0.2	1.1	0.16

5 t48CON	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	52	69	61	53	55	62	17	369	52.71
hpf2	28	27	58	67	42	60	50	332	47.43
hpf3	21	42	29	46	36	16	7	197	28.14
hpf4	7	21	17	25	38	5	5	118	16.86
hpf5	9	10	15	7	6	8	5	60	8.57
hpf6	11	2	14	28	6	5	1	67	9.57
hpf7	9	6	4	3	8	5	4	39	5.57
hpf8	2	3	6	2	2	5	3	23	3.29
hpf9	0	7	4	4	4	3	0	22	3.14
hpf10	0	3	4	3	2	1	0	13	1.86
total	139	190	212	238	199	170	92	1240	177.14
average	13.9	19	21.2	23.8	19.9	17	9.2	124	17.71

5 t48TNP	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	55	60	72	57	48	37	83	412	58.86
hpf2	3	19	46	25	8	6	67	174	24.86
hpf3	2	3	9	4	19	8	9	54	7.71
hpf4	3	2	2	0	2	5	0	14	2.00
hpf5	0	0	2	1	3	2	0	8	1.14
hpf6	0	0	2	3	1	2	0	8	1.14
hpf7	0	0	0	0	3	3	0	6	0.86
hpf8	0	0	0	0	0	0	0	0	0.00
hpf9	0	0	0	0	0	0	0	0	0.00
hpf10	0	0	0	0	0	0	0	0	0.00
total	63	84	133	90	84	63	159	676	96.57
average	6.3	8.4	13.3	9	8.4	6.3	15.9	67.6	9.66

Donor ID 8

8 toCON	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	1	0	0	0	0	0	0	1	0.14
hpf2	0	0	0	0	0	0	0	0	0.00
hpf3	0	0	0	0	0	0	1	1	0.14
hpf4	0	0	0	0	0	0	0	0	0.00
hpf5	0	0	0	0	0	0	0	0	0.00
hpf6	0	0	0	0	0	0	0	0	0.00
hpf7	0	0	0	0	0	1	0	1	0.14
hpf8	0	0	0	0	0	0	1	1	0.14
hpf9	0	0	0	0	0	0	0	0	0.00
hpf10	0	0	0	0	0	0	0	0	0.00
total	1	0	0	0	0	1	2	4	0.57
average	0.1	0	0	0	0	0.1	0.2	0.4	0.06

8 t48CON	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	131	110	55	129	72	78	123	698	99.71
hpf2	11	8	17	17	12	27	6	98	14.00
hpf3	5	6	3	4	9	6	0	33	4.71
hpf4	3	6	3	2	6	12	5	37	5.29
hpf5	3	4	4	0	2	6	4	23	3.29
hpf6	1	3	7	2	3	13	3	32	4.57
hpf7	12	7	5	2	5	1	5	37	5.29
hpf8	27	7	2	2	4	1	2	45	6.43
hpf9	8	2	3	12	55	3	0	83	11.86
hpf10	27	3	4	19	6	4	5	68	9.71
total	228	156	103	189	174	151	153	1154	164.86
average	22.8	15.6	10.3	18.9	17.4	15.1	15.3	115.4	16.49

8 t48TNP	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	72	37	47	69	31	59	55	370	52.86
hpf2	0	3	21	12	6	5	2	49	7.00
hpf3	4	3	3	0	3	1	8	22	3.14
hpf4	0	4	2	8	4	2	2	22	3.14
hpf5	1	2	3	9	3	2	0	20	2.86
hpf6	2	3	3	1	5	11	1	26	3.71
hpf7	1	1	8	4	4	13	4	35	5.00
hpf8	1	1	2	3	2	2	0	11	1.57
hpf9	1	4	0	0	1	0	0	6	0.86
hpf10	0	0	0	0	0	1	0	1	0.14
total	82	58	89	106	59	96	72	562	80.29
average	8.2	5.8	8.9	10.6	5.9	9.6	7.2	56.2	8.03

10.6 Cell counting data for burn wound biopsies

Burn ID A

A110CON	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	11	10	10	15	12	10	12	80	11.43
hpf2	8	13	5	13	9	10	11	69	9.86
hpf3	1	6	3	15	17	11	9	62	8.86
hpf4	5	7	11	5	3	15	3	49	7.00
hpf5	1	1	3	4	0	3	2	14	2.00
hpf6	1	1	1	0	0	0	0	3	0.43
hpf7	0	0	0	0	0	0	0	0	0.00
hpf8	0	0	0	0	0	0	0	0	0.00
hpf9	0	0	0	0	0	0	0	0	0.00
hpf10	0	0	0	0	0	0	0	0	0.00
total	27	38	33	52	41	49	37	277	39.57
average	2.7	3.8	3.3	5.2	4.1	4.9	3.7	27.7	3.96

A148CON	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	72	59	79	51	63	66	53	443	63.29
hpf2	18	23	41	42	13	25	30	192	27.43
hpf3	15	21	63	39	48	18	18	222	31.71
hpf4	22	12	28	24	54	6	21	167	23.86
hpf5	5	13	7	2	1	0	11	39	5.57
hpf6	0	2	3	4	1	6	9	25	3.57
hpf7	2	1	2	6	7	7	1	26	3.71
hpf8	3	2	3	7	12	6	0	33	4.71
hpf9	1	2	10	3	0	1	0	17	2.43
hpf10	0	13	0	0	0	0	0	13	1.86
total	138	148	236	178	199	135	143	1177	168.14
average	13.8	14.8	23.6	17.8	19.9	13.5	14.3	117.7	16.81

A148TNP	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	32	50	18	24	68	53	32	277	39.57
hpf2	8	13	13	16	28	16	10	104	14.86
hpf3	1	4	16	4	7	7	6	45	6.43
hpf4	1	3	3	5	6	11	4	33	4.71
hpf5	1	0	3	7	3	14	7	35	5.00
hpf6	1	3	1	1	0	0	0	6	0.86
hpf7	0	0	3	2	1	0	0	6	0.86
hpf8	1	0	0	1	2	0	0	4	0.57
hpf9	0	0	0	1	0	0	0	1	0.14
hpf10	0	1	0	0	0	0	0	1	0.14
total	45	74	57	61	115	101	59	512	73.14
average	4.5	7.4	5.7	6.1	11.5	10.1	5.9	51.2	7.31

Burn ID Ba

B15CON	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	27	47	42	39	36	4	8	203	29.00
hpf2	22	23	28	18	15	3	10	119	17.00
hpf3	20	10	42	42	15	2	4	135	19.29
hpf4	8	7	12	10	0	1	0	38	5.43
hpf5	7	13	3	4	7	25	25	84	12.00
hpf6	3	1	2	0	17	25	3	51	7.29
hpf7	23	6	3	9	3	3	1	48	6.86
hpf8	24	4	3	0	0	0	0	31	4.43
hpf9	0	2	0	0	0	0	0	2	0.29
hpf10	5	1	1	0	3	0	0	10	1.43
total	139	114	136	122	96	63	51	721	103.00
average	13.9	11.4	13.6	12.2	9.6	6.3	5.1	72.1	10.30

B130CON	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	75	85	74	83	79	76	95	567	81.00
hpf2	12	8	19	12	7	10	11	79	11.29
hpf3	10	17	13	14	32	16	19	121	17.29
hpf4	24	25	24	12	21	35	19	160	22.86
hpf5	25	24	21	23	31	71	19	214	30.57
hpf6	24	28	37	31	45	41	19	225	32.14
hpf7	15	41	20	29	37	21	19	182	26.00
hpf8	16	21	34	40	24	42	19	196	28.00
hpf9	13	33	80	58	19	34	19	256	36.57
hpf10	16	34	70	40	24	12	19	215	30.71
total	230	316	392	342	319	358	19	1976	282.29
average	23	31.6	39.2	34.2	31.9	35.8	1.9	221.5	31.64

B130TNP	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	42	52	22	21	46	86	90	359	51.29
hpf2	24	12	8	11	17	17	15	104	14.86
hpf3	5	7	3	16	18	21	20	90	12.86
hpf4	10	1	20	16	17	22	23	109	15.57
hpf5	4	3	14	22	35	43	28	149	21.29
hpf6	0	11	23	28	23	59	15	159	22.71
hpf7	7	17	25	22	25	27	6	129	18.43
hpf8	73	39	10	0	12	4	0	138	19.71
hpf9	19	78	19	0	0	0	0	116	16.57
hpf10	1	0	1	18	24	0	0	44	6.29
total	185	220	145	154	217	279	197	1397	199.57
average	18.5	22	14.5	15.4	21.7	27.9	19.7	139.7	19.96

Burn ID Bb

C15CON	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	6	33	64	72	62	32	13	282	40.29
hpf2	5	6	9	44	28	10	5	107	15.29
hpf3	1	13	26	36	17	7	3	103	14.71
hpf4	5	43	14	27	3	5	4	101	14.43
hpf5	10	34	47	20	2	5	0	118	16.86
hpf6	7	13	2	43	20	16	0	101	14.43
hpf7	5	0	3	5	22	8	0	43	6.14
hpf8	2	0	0	1	2	0	0	5	0.71
hpf9	0	0	0	0	1	0	2	3	0.43
hpf10	0	0	0	0	6	1	0	7	1.00
total	41	142	165	248	163	84	27	870	124.29
average	4.1	14.2	16.5	24.8	16.3	8.4	2.7	87	12.43

C130CON	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	100	69	115	163	186	183	177	993	141.86
hpf2	34	24	18	15	18	18	19	146	20.86
hpf3	14	15	13	31	38	21	26	158	22.57
hpf4	23	29	13	24	25	20	17	151	21.57
hpf5	17	10	7	18	25	16	26	119	17.00
hpf6	7	1	11	23	23	11	19	95	13.57
hpf7	11	15	23	19	79	16	35	198	28.29
hpf8	5	12	17	7	43	49	31	164	23.43
hpf9	0	1	3	11	9	10	12	46	6.57
hpf10	2	2	3	6	3	8	0	24	3.43
total	213	178	223	317	449	352	362	2094	299.14
average	21.3	17.8	22.3	31.7	44.9	35.2	36.2	209.4	29.91

C130TNP	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	39	23	33	39	35	19	18	206	29.43
hpf2	4	5	15	11	9	17	13	74	10.57
hpf3	3	5	5	5	9	8	14	49	7.00
hpf4	6	7	3	8	7	9	14	54	7.71
hpf5	14	7	5	4	22	28	9	89	12.71
hpf6	7	3	0	3	3	6	4	26	3.71
hpf7	7	3	3	7	2	11	4	37	5.29
hpf8	5	6	7	3	7	2	0	30	4.29
hpf9	9	12	0	5	2	0	0	28	4.00
hpf10	4	29	4	1	2	0	0	40	5.71
total	98	100	75	86	98	100	76	633	90.43
average	9.8	10	7.5	8.6	9.8	10	7.6	63.3	9.04

Burn ID Ca

D110CON	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	6	9	19	24	11	15	10	94	13.43
hpf2	4	7	4	6	10	3	3	37	5.29
hpf3	1	0	5	0	4	3	1	14	2.00
hpf4	1	0	0	0	0	0	1	2	0.29
hpf5	1	0	0	0	0	0	1	2	0.29
hpf6	0	0	0	0	0	0	0	0	0.00
hpf7	0	0	0	0	0	0	0	0	0.00
hpf8	0	0	0	0	0	0	0	0	0.00
hpf9	0	0	0	0	0	0	0	0	0.00
hpf10	0	0	0	0	0	0	0	0	0.00
total	13	16	28	30	25	21	16	149	21.29
average	1.3	1.6	2.8	3	2.5	2.1	1.6	14.9	2.13

D148CON	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	27	6	7	9	4	5	5	63	9.00
hpf2	3	5	1	22	0	5	2	38	5.43
hpf3	1	0	5	1	2	1	2	12	1.71
hpf4	0	2	0	3	0	1	1	7	1.00
hpf5	0	1	0	2	0	0	0	3	0.43
hpf6	0	0	1	1	1	0	3	6	0.86
hpf7	1	0	2	0	0	0	0	3	0.43
hpf8	2	0	0	0	0	1	0	3	0.43
hpf9	0	0	0	0	0	0	1	1	0.14
hpf10	0	0	0	0	0	0	0	0	0.00
total	34	14	16	38	7	13	14	136	19.43
average	3.4	1.4	1.6	3.8	0.7	1.3	1.4	13.6	1.94

D148TNP	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	10	8	8	12	18	19	24	99	14.14
hpf2	6	3	7	11	3	9	12	51	7.29
hpf3	0	1	3	2	2	13	4	25	3.57
hpf4	2	2	4	2	0	1	0	11	1.57
hpf5	0	1	4	2	0	1	0	8	1.14
hpf6	0	5	0	2	0	0	0	7	1.00
hpf7	0	0	2	0	1	0	0	3	0.43
hpf8	0	0	0	0	0	0	0	0	0.00
hpf9	0	0	0	0	0	0	0	0	0.00
hpf10	0	0	0	0	0	0	0	0	0.00
total	18	20	28	31	24	43	40	204	29.14
average	1.8	2	2.8	3.1	2.4	4.3	4	20.4	2.91

Burn ID Cb

E110CON	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	16	25	35	18	22	18	14	148	21.14
hpf2	13	59	19	25	16	14	4	150	21.43
hpf3	4	13	42	23	10	0	4	96	13.71
hpf4	0	0	3	2	6	1	0	12	1.71
hpf5	0	0	2	1	4	0	0	7	1.00
hpf6	2	0	0	0	0	0	0	2	0.29
hpf7	0	0	0	0	0	0	0	0	0.00
hpf8	2	0	0	0	0	0	0	2	0.29
hpf9	2	0	0	0	0	0	0	2	0.29
hpf10	0	0	0	0	0	0	0	0	0.00
total	39	97	101	69	58	33	22	419	59.86
average	3.9	9.7	10.1	6.9	5.8	3.3	2.2	41.9	5.99

E148CON	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	6	5	28	20	3	12	25	99	14.14
hpf2	3	2	2	4	12	29	6	58	8.29
hpf3	2	1	0	7	6	19	0	35	5.00
hpf4	0	1	0	0	2	5	2	10	1.43
hpf5	0	1	0	0	11	19	1	32	4.57
hpf6	1	1	0	0	3	3	2	10	1.43
hpf7	0	0	0	0	0	3	2	5	0.71
hpf8	0	0	0	0	1	1	0	2	0.29
hpf9	0	1	0	0	0	1	0	2	0.29
hpf10	0	0	0	0	0	2	0	2	0.29
total	12	12	30	31	38	94	38	255	36.43
average	1.2	1.2	3	3.1	3.8	9.4	3.8	25.5	3.64

E148TNP	col. 1	col. 2	col. 3	col. 4	col. 5	col. 6	col. 7	totals	averages
hpf1	10	17	2	17	19	28	22	115	16.43
hpf2	2	1	1	6	6	4	8	28	4.00
hpf3	0	1	2	1	1	2	1	8	1.14
hpf4	4	0	2	0	0	0	0	6	0.86
hpf5	4	0	0	0	0	0	0	4	0.57
hpf6	0	0	3	0	0	0	0	3	0.43
hpf7	0	0	0	1	1	0	1	3	0.43
hpf8	0	2	0	0	1	0	0	3	0.43
hpf9	0	0	0	0	0	0	0	0	0.00
hpf10	0	0	0	0	0	0	0	0	0.00
total	20	21	10	25	28	34	32	170	24.29
average	2	2.1	1	2.5	2.8	3.4	3.2	17	2.43

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